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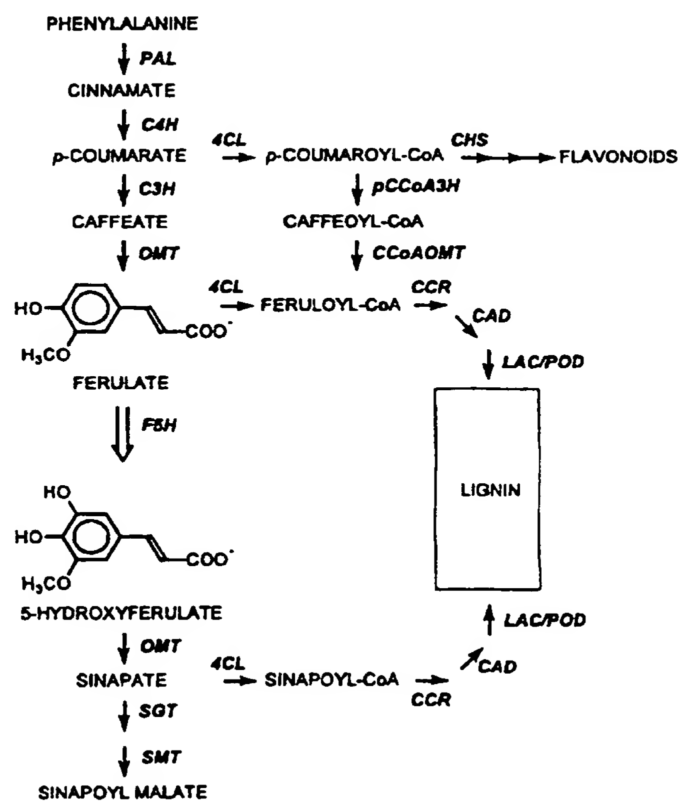
(54) Title: MANIPULATION OF LIGNIN COMPOSITION IN PLANTS USING A TISSUE-SPECIFIC PROMOTER

(57) Abstract

The present invention relates to methods and materials in the field of molecular biology, the manipulation of the phenylpropanoid pathway and the regulation of protein synthesis through plant genetic engineering. More particularly, the invention relates to the introduction of a foreign nucleotide sequence into a plant genome, wherein the introduction of the nucleotide sequence effects an increase in the syringyl content of the plant's lignin. In one specific aspect, the invention relates to methods for modifying the plant lignin composition in a plant cell by the introduction thereinto of a foreign nucleotide sequence comprising a tissue specific plant promoter sequence and a sequence encoding an active ferulate-5-hydroxylase (F5H) enzyme. Plant transformants harboring an inventive promoter-F5H construct demonstrate increased levels of syringyl monomer residues in their lignin, rendering the polymer more readily delignified and, thereby, rendering the plant more readily pulped or digested.



MANIPULATION OF LIGNIN COMPOSITION IN PLANTS USING A TISSUE-SPECIFIC PROMOTER

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REFERENCES TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/022,288, filed July 19, 1996, and U.S. Provisional Application No. 60/032,908, filed December 16, 1996, each of which is hereby incorporated by reference herein in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to methods and materials in the field of molecular biology and the regulation of protein synthesis through plant genetic engineering. More particularly, the invention relates to the introduction of a foreign nucleotide sequence into a plant genome, wherein the introduction of the nucleotide sequence effects an increase in the syringyl content of lignin synthesized by the plant. Specifically, the invention relates in one aspect to methods for modifying the lignin composition in a plant cell by the introduction thereinto of a foreign nucleotide sequence comprising a tissue-specific plant promoter sequence and a coding sequence encoding an active ferulate-5-hydroxylase (F5H) enzyme. Plant transformants harboring an inventive promoter-F5H construct demonstrate increased levels of syringyl monomer residues in lignin synthesized thereby, rendering the polymer more readily delignified and, thereby, rendering the plant more readily pulped or digested.

Discussion of Related Art

Lignin is one of the major products of the general phenylpropanoid pathway, set forth in Figure 1. and is one of the most abundant organic molecules in the biosphere (Crawford. (1981) Lignin Biodegradation and Transformation. New York: John Wiley and Sons). Referring to Figure 1. lignin biosynthesis via the phenylpropanoid biosynthetic pathway is initiated by the conversion of phenylalanine into cinnamate through the action of phenylalanine ammonia lyase (PAL). The second enzyme of the pathway is cinnamate-4-hydroxylase (C4H), a cytochrome P450-dependent monooxygenase (P450) which is responsible for the conversion of cinnamate to *p*-coumarate. The second hydroxylation of the pathway is catalyzed by a relatively ill-characterized enzyme, *p*-coumarate-3-hydroxylase (C3H). whose product is caffeic acid. Caffeic acid is subsequently *O*-methylated by caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT) to form ferulic acid, a direct precursor of lignin. The last hydroxylation reaction of the general phenylpropanoid pathway is catalyzed by F5H. The 5-hydroxyferulate produced by F5H is then *O*-methylated by OMT, the same enzyme that carries out the *O*-methylation of caffeic acid. This dual specificity of OMT has been confirmed by the cloning of the OMT gene, and expression of the protein in *E. coli* (Bugos et al., Plant Mol. Biol. 17, 1203, (1991); Gowri et al., (1991) Plant Physiol., 97, 7, (1991)).

Recently, a different route for the biosynthesis of lignin monomers has received attention (Kneusel et al., Arch. Biochem. Biophys. 269, 455, (1989); Kühnl et al., Plant Science 60, 21, (1989); Pakusch et al., Arch. Biochem. Biophys. 271, 488, (1989); Pakusch et al., Plant Physiol. 95, 137, (1991); Schmitt et al., Jour. Biol. Chem. 266, 17416, (1991); Ye et al., Plant Cell 6, 1427, (1994); Ye and Varner. Plant Physiol. 108, 459, (1995)). This so-called "alternative" pathway involves the activation of *p*-coumaric acid to its coenzyme A thioester, followed by hydroxylation and methylation reactions that generate feruloyl-CoA as the product of the pathway. Considering that ferulic acid can also be synthesized by the free acid pathway and can be activated to its CoA thioester by (hydroxy)cinnamoyl CoA ligase (4CL), lignin monomer biosynthesis probably occurs via a cross-linked network of pathways. Indeed, the continued accumulation of guaiacyl lignin in OMT suppressed plants (Atanassova et al., Plant J. 8, 465, (1995) 1995; Van

Doorsselaere et al., Plant J. 8, 855, (1995)) indicates that the alternative pathway may be a major contributor to lignin biosynthesis in woody plants. Both the conventional "free acid" pathway and the "alternative" pathway have been reported to be developmentally regulated, providing different routes for the synthesis of lignin monomers in different cell types (Ye and Varner, *supra*). This differential gene regulation may be one of the mechanisms by which lignin monomer composition is controlled.

The committed steps of lignin biosynthesis are catalyzed by (hydroxy)cinnamoyl CoA reductase (CCR) and (hydroxy)cinnamoyl alcohol dehydrogenase (CAD), which ultimately generate coniferyl alcohol from ferulic acid and sinapoyl alcohol from sinapic acid. Coniferyl alcohol and sinapoyl alcohol are polymerized by extracellular oxidases to yield guaiacyl lignin and syringyl lignin respectively, although syringyl lignin is more accurately described as a co-polymer of both monomers.

Although ferulic acid, sinapic acid, and in some cases *p*-coumaric acid are channeled into lignin biosynthesis, in some plants these compounds are precursors for soluble secondary metabolites. For example, in *Arabidopsis*, sinapic acid serves as a precursor for lignin biosynthesis but it is also channeled into the synthesis of soluble sinapic acid esters. In this pathway, sinapic acid is converted to sinapoylglucose which serves as an intermediate in the biosynthesis of sinapoylmalate (Figure 1). Sinapic acid and its esters are fluorescent and may be used as a marker of plants deficient in those enzymes needed to produce sinapic acid (Chapple et al., Plant Cell 4, 1413, (1992)).

In nature, lignification, or integration of lignin into the plant secondary cell wall, provides rigidity and structural integrity to wood and is in large part responsible for the structural integrity of tracheary elements in a wide variety of plants, giving them the ability to withstand tension generated during transpiration. Lignin also imparts decay resistance to the plant secondary cell wall and is thought to have been essential to the evolution of terrestrial plants. Lignin is well suited to these capacities because of its physical characteristics and its resistance to biochemical degradation. Unfortunately, this same resistance to degradation has a significant impact on the utilization of

lignocellulosic plant material (Whetten et al., Forest Ecol. Management 43, 301, (1991)).

In angiosperms, lignin is composed mainly of two aromatic monomers which differ in their methoxyl substitution pattern. As described above, precursors for lignin biosynthesis are synthesized from L-phenylalanine via the phenylpropanoid pathway which provides ferulic acid (4-hydroxy-3-methoxycinnamic acid) and sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) for the synthesis of guaiacyl- and syringyl-substituted lignin monomers, respectively. Two cytochrome P450-dependent monoxygenases (P450s) are required for the synthesis of lignin monomers. C4H catalyzes the second step of the phenylpropanoid pathway, the hydroxylation of the aromatic ring of cinnamic acid at the *para* position, and its activity is required for the biosynthesis of all lignin precursors. Ferulate-5-hydroxylase (F5H) catalyzes the *meta*-hydroxylation of ferulic acid in the monomer-specific pathway branch required for sinapic acid and syringyl lignin biosynthesis.

The balance between guaiacyl and syringyl units in lignin varies between plant species, within a given plant, and even within the wall of a single plant cell. For example, the lignin of the mature *Arabidopsis* rachis (flowering stem) contains guaiacyl and syringyl residues in an overall ratio of approximately 4:1; however, this ratio is not constant throughout plant development. The syringyl content of the rachis increases from less than 6 mol% within the apical 4 cm of the bolt to over 26 mol% near the base of the inflorescence. Histochemical staining of *Arabidopsis* rachis cross-sections indicates that syringyl lignin biosynthesis is also developmentally regulated in a tissue-specific manner. Accumulation of syringyl lignin (i.e., lignin synthesized from syringyl and guaiacyl monomers) is restricted to the cells of the sclerified parenchyma that flank the vascular bundles while guaiacyl lignin (i.e. lignin synthesized from guaiacyl monomers only) is deposited only in the cells of the vascular bundle. The increase in syringyl lignin content during rachis development is a consequence of sclerified parenchyma maturation as these cells undergo secondary thickening after the vascular bundle has been formed from the cells of the procambium.

The monomeric composition of lignin has significant effects on its chemical degradation during industrial pulping (Chiang et al., Tappi, 71, 173, (1988). The guaiacyl lignins (derived from ferulic acid) characteristic of softwoods such as pine, require substantially more alkali and longer

incubations during pulping in comparison to the guaiacyl-syringyl lignins (derived from ferulic acid and sinapic acid) found in hardwoods such as oak. The reasons for the differences between these two lignin types has been explored by measuring the degradation of model compounds such as guaiacylglycerol-guaiacyl ether, syringylglycerol-guaiacyl ether, and syringylglycerol-(4-methylsyringyl) ether (Kondo et al., Holzforschung, 41, 83, (1987)) under conditions that mimic those used in the pulping process. In these experiments, the mono- and especially di-syringyl compounds were cleaved three to fifteen times faster than their corresponding diguaiacyl homologues. These model studies are in agreement with studies comparing the pulping of Douglas fir and sweetgum wood where the major differences in the rate of pulping occurred above 150 C where arylglycerol-aryl ether linkages were cleaved (Chiang and Funaoka, Holzforschung, 44, 309, (1990)).

Another factor affecting chemical degradation of the two lignin forms may be the condensation of lignin-derived guaiacyl and syringyl residues to form diphenylmethane units. The presence of syringyl residues in hardwood lignins leads to the formation of syringyl-containing diphenylmethane derivatives that remain soluble during pulping, while the diphenylmethane units produced during softwood pulping are alkali-insoluble and thus remain associated with the cellulosic products (Chiang et al., Holzforschung, 44, 147, (1990); Chiang and Funaoka, *supra*). Further, it is thought that the abundance of 5-5'-diaryl crosslinks that can occur between guaiacyl residues contributes to resistance to chemical degradation. This linkage is resistant to alkali cleavage and is much less common in lignin that is rich in syringyl residues because of the presence of the 5-O-methyl group in syringyl residues. Thus, the incorporation of syringyl residues results in what is known as "non-condensed lignin", a polymer that is significantly easier to pulp than condensed lignin.

Similarly, lignin composition and content in grasses is a major factor in determining the digestibility of lignocellulosic materials that are fed to livestock (Jung, H.G. & Deetz, D.A. (1993) Cell wall lignification and degradability in Forage Cell Wall Structure and Digestibility (H.G. Jung, D.R. Buxton, R.D. Hatfield, and J. Ralph eds.), ASA/CSSA/SSSA Press, Madison, WI.). The

incorporation of the lignin polymer into the plant cell wall prevents microbial enzymes from having access to the cell wall polysaccharides that make up the plant cell wall. As a result. these polysaccharides are substantially unavailable for digestion by livestock. and much of the valuable carbohydrates contained within animal feedstock passes through the animals undigested. Thus. an increase in the dry matter of grasses over the growing season is counteracted by a decrease in digestibility caused principally by increased cell wall lignification. In light of the above background. it is clear that biotechnological modification or manipulation of lignin monomer composition is economically desirable. as it provides the ability to significantly decrease the cost of pulp production and to increase the nutritional value of animal feedstocks thereby also enhancing their economic value.

The mechanism(s) by which plants control lignin monomer composition has been the subject of much speculation. As mentioned above. gymnosperms do not synthesize appreciable amounts of syringyl lignin. In angiosperms. syringyl lignin deposition is developmentally regulated: primary xylem contains guaiacyl lignin. while the lignin of secondary xylem and sclerenchyma is guaiacyl-syringyl lignin (Venverloo. Holzforschung 25. 18 (1971); Chapple et al.. *supra*). No plants have been found to contain purely syringyl lignin. It is still not clear how this specificity is controlled: however. a number of enzymatic steps have previously been proposed as sites for the control of lignin monomer composition and at least five possible enzymatic control sites exist. namely OMT. F5H, 4CL. CCR. and CAD. For example. the substrate specificities of OMT (Shimada et al., Phytochemistry. 22. 2657. (1972); Shimada et al., Phytochemistry. 12. 2873. (1973); Gowri et al.. *supra*; Bugos et al.. *supra*) and CAD (Sarni et al.. Eur. J. Biochem.. 139. 259. (1984); Goffner et al.. Planta.. 188. 48. (1992); O'Malley et al.. Plant Physiol., 98. 1364. (1992)) are correlated with the differences in lignin monomer composition seen in gymnosperms and angiosperms. and the expression of 4CL isozymes (Grand et al.. Physiol. Veg. 17. 433. (1979); Grand et al., Planta., 158, 225. (1983)) has been suggested to be related to the tissue specificity of lignin monomer composition seen in angiosperms.

Although there are at least five possible enzyme targets. much attention has been directed

recently to investigating the use of OMT and CAD to manipulate lignin monomer composition in transgenic plants (Dwivedi et al.. Plant Mol. Biol. 26. 61, (1994); Halpin et al.. Plant J. 6. 339. (1994); Ni et al.. Transgen. Res. 3. 120 (1994); Atanassova et al., *supra*; Van Doorsselaere et al.. *supra*). Most of these studies have focused on sense and antisense suppression of OMT expression. This approach has met with variable results. probably owing to the degree of OMT suppression achieved in the various studies. The most dramatic effects were seen by using homologous OMT constructs to suppress OMT expression in tobacco (Atanassova et al.. *supra*) and poplar (Van Doorsselaere et al.. *supra*). Both of these studies found that as a result of transgene expression, there was a decrease in the content of syringyl lignin and a concomitant appearance of 5-hydroxyguaiacyl residues. As a result of these studies. Van Doorsselaere et al.. (WO 9305160) disclose a method for the regulation of lignin biosynthesis through the genomic incorporation of an OMT gene in either the sense of anti-sense orientation. In contrast. Dixon et al. (WO 9423044) demonstrate the reduction of lignin content in plants transformed with an OMT gene. rather than a change in lignin monomer composition.

Similar research has focused on the suppression of CAD expression. The conversion of coniferaldehyde and sinapaldehyde to their corresponding alcohols in transgenic tobacco plants has been modified with the incorporation of an *A. cordata* CAD gene in anti-sense orientation (Hibino et al.. Biosci. Biotechnol. Biochem.. 59. 929. (1995)). A similar effort aimed at antisense inhibition of CAD expression generated a lignin with increased aldehyde content. but only a modest change in lignin monomer composition (Halpin et al.. *supra*). This research has resulted in the disclosure of methods for the reduction of CAD activity using sense and anti-sense expression of a cloned CAD gene to effect inhibition of endogenous CAD expression in tobacco [Boudet et al., (U.S. 5.451.514) and Walter et al.. (WO 9324638); Bridges et al.. (CA 2005597)]. None of these strategies. however. increased the syringyl content of lignin. a trait that is correlated with improved digestibility and chemical degradability of lignocellulosic material (Chiang et al.. *supra*. Chiang and Funaoka. *supra*; Jung et al.. *supra*).

In view of this background. the present invention involves producing transformed plants

having increased levels of syringyl residues in their lignin to facilitate chemical degradation of the lignin. Increased syringyl content in lignin produced by a plant transformed in accordance with the invention is achieved by modifying the enzyme pathway responsible for the production of lignin monomers in a manner distinct from those attempted previously. Specifically, this result is achieved in one preferred aspect of the invention by eliciting over-expression of the enzyme F5H in plant cells undergoing lignin synthesis. The term "expression", as used herein, refers to the production of the protein product encoded by a nucleotide coding sequence. "Over-expression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

Although F5H is a key enzyme in the biosynthesis of syringyl lignin monomers it has not been exploited to date in efforts to engineer lignin quality. In fact, since the time of its discovery over 30 years ago (Higuchi et al., Can. J. Biochem. Physiol., 41, 613, (1963)) there has been only one demonstration of the activity of F5H published (Grand, C., FEBS Lett. 169, 7, (1984)). Grand demonstrated that F5H from poplar was a cytochrome P450-dependent monooxygenase (P450) as analyzed by the classical criteria of dependence on NADPH and light-reversible inhibition by carbon monoxide. Grand further demonstrated that F5H is associated with the endoplasmic reticulum of the cell. The lack of attention given to F5H in recent years may be attributed in general to the difficulties associated with dealing with membrane-bound enzymes, and specifically to the liability of F5H when treated with the detergents necessary for solubilization (Grand, *supra*). The most recent discovery surrounding F5H has been made by Chapple et al., (*supra*) who reported a mutant of *Arabidopsis thaliana* L. Heynh named *fah1* that is deficient in the accumulation of sinapic acid-derived metabolites, including the guaiacyl-syringyl lignin typical of angiosperms. This locus, termed *FAH1*, encodes F5H.

In spite of sparse information about F5H in the published literature, the present inventor has been successful in the isolation, cloning, and sequencing of the F5H gene (Meyer et al., Proc. Natl. Acad. Sci. USA 93, 6869, (1996)). The present inventor has also demonstrated that the stable integration of the F5H gene into the plant genome, where the expression of the F5H gene is under

the control of a promoter other than the gene's endogenous promoter (such as. for example. the 35S promoter). leads to an altered regulation of lignin biosyntheses. It has been determined that causing over-expression of the enzyme F5H in Arabidopsis using the 35S promoter allows the plant to produce lignin containing up to 30% of the syringyl monomer. This over-expression may be accomplished by constructing a 35S promoter/F5H construct and transforming a plant host with the construct. Similarly. over-expression of the enzyme F5H in tobacco using the 35S promoter allows the plant to produce lignin in its petioles (leaf stems) containing up to 40% of the syringyl monomer. One problem with this system. however. is that Arabidopsis plants transformed with the construct are unable to produce lignin having a syringyl content greater than about 30 mol%. Similarly. in tobacco plants transformed with the 35S promoter/F5H construct. no change was observed in the syringyl monomer content of stem lignin which is naturally approximately 50%.

These limitations are overcome by the present invention. which provides in one preferred aspect a genetic construct assembled from a tissue-specific promoter sequence endogenous to plant cells and a nucleotide sequence which encodes the enzyme F5H. The construct may be used to transform plants. thereby providing transformed plants capable of producing lignin having a syringyl content greater than a native plant. For example. an Arabidopsis plant may be transformed in accordance with the invention such that the transformed Arabidopsis plant is capable of producing lignin having a syringyl content of greater than about 30 mol%. Furthermore. inventive constructs may be used to transform a tobacco plant such that the transformed tobacco plant is capable of producing lignin in its petioles having a syringyl content of greater than about 40 mol% and such that the transformed tobacco plant is capable of producing stem lignin having a syringyl content of greater than about 50 mol%.

SUMMARY OF THE INVENTION

The present invention relates to the isolation. purification and use of DNA constructs comprising a tissue-specific plant promoter. for example. a C4H promoter. and a nucleotide sequence useful for the modification of lignin biosynthesis such as. for example. an F5H coding sequence. Inventive DNA constructs employing lignification-specific promoters such as the C4H promoter are useful for modifying the quality or quantity of a plants lignin. and specific examples of constructs are provided herein for increasing the syringyl content of a plant's lignin by targeting over-expression of the F5H enzyme to plant cells producing lignin or providing the precursors for lignin biosynthesis. Lignification-specific promoters set forth in Figure 1. such as the C4H promoter are effective in directing gene expression to lignifying cells. and are thus useful promoters for modifying gene expression in these cells via antisense or co-suppression technologies. As discussed in the Background above and set forth in Figure 1. the F5H enzyme catalyzes an irreversible hydroxylation step that diverts ferulic acid away from guaiacyl lignin biosynthesis and toward sinapic acid and syringyl lignin biosynthesis. Specifically. F5H catalyzes the reaction of ferulate to 5-hydroxyferulate and over-expression thereof in the proper plant tissues under the control of lignification-specific promoters such as the C4H promoter results in synthesis of lignin having a high syringyl content. i.e. greater than that achieved in prior art plants of the same species.

High syringyl lignins are more readily degraded during the pulping process and during ruminant digestion of lignocellulosic feedstocks. The unaltered morphology of tracheary elements and sclerified parenchyma in transgenic plants depositing lignin highly enriched in syringyl units suggests that this lignin still provides lignified cells with sufficient rigidity to function normally in water conduction and mechanical support. Thus. a surprisingly advantageous result is achieved in accordance with the invention upon increasing the syringyl content of crop species and trees. thereby generating lignins that are easier to digest or extract without detrimental consequences on agricultural performance.

It is presently shown that inventive DNA constructs may advantageously be used according to the invention to transform a plant. thereby providing an inventive transformed plant which produces lignin having a syringyl:guaiacyl ratio that is greater than that of a non-transformed plant of the same

species or a plant of the same species transformed using constructs known in the prior art. The present invention thus provides methods for genetically engineering plants to provide inventive transformed plants which may be readily delignified. The invention features DNA constructs comprising a tissue-specific plant promoter sequence and a coding sequence as set forth herein, as well as DNA constructs comprising nucleotide sequences having substantial identity thereto and having similar levels of functionality. Inventive constructs may be inserted into an expression vector to produce a recombinant DNA expression system which is also an aspect of the invention.

In a preferred aspect of the invention, there is provided an isolated nucleic-acid construct comprising a nucleotide sequences which correspond to a regulatory sequence of the C4H genomic sequence set forth in SEQ ID NO:1 and a nucleotide sequence having substantial similarity to the sequence set forth in either SEQ ID NO:2 (F5H genomic nucleotide sequence) or SEQ ID NO:3 (F5H cDNA). In a preferred aspect of the invention, the enzyme encoded thereby preferably has an amino acid sequence having substantial identity to the F5H enzyme set forth in SEQ ID NO:4, wherein the amino acid sequence may include amino acid substitutions, additions and deletions that do not alter the function of the F5H enzyme.

It is an object of the present invention to provide an isolated DNA construct which comprises a tissue-specific promoter and a nucleotide sequence encoding an F5H enzyme, the construct finding advantageous use when incorporated into a vector or plasmid as a transformant for a plant.

Additionally, it is an object of the invention to provide transformed plants which produce lignin having a syringyl content greater than a native plant of the same species, thereby providing resources for the pulping industry which are much more readily and economically delignified, and providing agricultural feedstocks which are much more readily and efficiently digested by livestock.

Further objects, advantages and features of the present invention will be apparent from the detailed description herein.

BRIEF DESCRIPTION OF THE FIGURES

Although the characteristic features of this invention will be particularly pointed out in the claims, the invention itself, and the manner in which it may be made and used, may be better understood by referring to the following description taken in connection with the accompanying figures forming a part hereof.

Figure 1 illustrates the general phenylpropanoid pathway, and associated pathways leading to lignin, sinapate esters, and flavonoids in *Arabidopsis*. The structures of ferulate and 5-hydroxyferulate are shown to emphasize the reaction catalyzed by ferulate-5-hydroxylase (F5H). The names of enzymes are shown in italics and include phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), *p*-coumarate-3-hydroxylase (C3H), caffeic acid/5-hydroxyferulic acid)-methyltransferase (OMT), sinapic acid:UDPG sinapoyltransferase (SGT), sinapoyl glucose:malate sinapoyltransferase (SMT), hydroxycinnamoyl-CoA ligase (4CL), *p*-coumaroyl-CoA-3-hydroxylase (*p*CCoA3H), caffeoyl-CoA*O*-methyltransferase (CCoAOMT), hydroxycinnamoyl-CoA reductase (CCR), hydroxycinnamoyl alcohol dehydrogenase (CAD), laccase/peroxidase (LAC/POD) and chalcone synthase (CHS).

Figure 2 illustrates a Southern blot analysis comparing hybridization of the F5H cDNA to *Eco*RI digested genomic DNA isolated from wild type *Arabidopsis thaliana* and a number of *fah1* mutants.

Figure 3 is a Northern blot analysis comparing hybridization of the F5H cDNA to RNA isolated from wild type *Arabidopsis thaliana* and a number of *fah1* mutants.

Figure 4 is an illustration of the pBIC20-F5H cosmid, as well as the the F5H overexpression constructs pGA482-35S-F5H and pGA482-C4H-F5H in which the F5H gene is expressed under the control of the constitutive cauliflower mosaic virus 35S promoter, or the *Arabidopsis thaliana* C4H promoter, respectively.

Figure 5 shows an analysis of sinapic acid-derived secondary metabolites in wild type, the *fahl-2* mutant, and independently-derived transgenic *fahl-2* plants carrying the T-DNA derived from the pBIC20-F5H cosmid, or the pGA482-35S-F5H overexpression construct.

Figure 6 shows Southern blot analysis of the C4H locus in Arabidopsis. The C4H cDNA was used as a probe against DNA isolated from the Columbia ecotype digested with *Csp*45I, *Hin*cII, *Hin*dIII, *Nde*I, and *Xma*I. DNA from both Columbia and Landsberg *erecta* ecotypes digested with *Sty*I was included to illustrate the restriction fragment length polymorphism identified with this enzyme.

Figure 7 shows *in vivo* GUS staining in C4H-GUS transformants. A, 10 day-old seedling; B, 10 day-old seedling root; C, mature leaf; D, rachis transverse section; E, flower; F, mature leaf stained 48 hours after wounding; G, mature leaf stained immediately after wounding. A, C, E, F, G, Bar = 500 μm. B, C, Bar = 10 μm.

Figure 8 shows the impact of 35S promoter-driven F5H overexpression on lignin monomer composition. Stem tissue from five week old plants of the wild type, the *fah1-2* mutant, and nine independent *fah1-2* lines homozygous for the 35S-F5H transgene (top) were harvested and used for RNA isolation and the determination of lignin monomer composition. Blots were probed with the F5H cDNA and were exposed to film for 24 hours to visualize the level of F5H expression in the wild type and the *fah1-2* mutant (left panel), and for two hours to evaluate F5H expression in the 35S-F5H transgenics (right panel). Lignin monomer composition of total stem tissue was determined for each line by nitrobenzene oxidation. Average values of ten replicates and their standard deviations are shown (bottom).

Figure 9 shows histochemical staining for lignin monomer composition in Arabidopsis stem cross sections. Lower rachis segments were hand sectioned, stained with the Mäule reagent and observed by light microscopy using cross-polarizing optics. Red staining indicates the presence of syringyl residues in the plant secondary cell wall.

Figure 10 shows the impact of C4H promoter-driven F5H overexpression on lignin monomer composition. Stem tissue from five week old plants of the wild type, the *fah1-2* mutant, and nine independent *fah1-2* lines homozygous for the C4H-F5H transgene (top) were harvested and used for RNA isolation and the determination of lignin monomer composition. Blots were probed with the F5H cDNA and were exposed to film for 12 hours to visualize the level of F5H

expression. Lignin monomer composition of total stem tissue was determined for each line by nitrobenzene oxidation. Average values of five replicates and their standard deviations are shown (bottom).

Figure 11 shows a GC analysis of lignin nitrobenzene oxidation products to illustrate the impact of F5H overexpression on lignin monomer composition in the wild type, the *fah1-2* mutant, and the *fah1-2* mutant carrying the T-DNA derived from the 35S-F5H overexpression construct, or the C4H-F5H overexpression construct.

DETAILED DESCRIPTION OF THE INVENTION

For purposes of promoting an understanding of the principles of the invention, reference will now be made to particular embodiments of the invention and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications in the invention, and such further applications of the principles of the invention as described herein being contemplated as would normally occur to one skilled in the art to which the invention pertains.

The present invention relates to DNA constructs that may be integrated into a plant to provide an inventive transformed plant which over-expresses F5H or another key lignin biosynthesis enzyme, in lignin-producing cells. Over-expression of F5H results in an increased conversion of ferulic acid to sinapic acid, and results in an increase in the syringyl content of the lignin polymer produced by the plant. The present inventor has discovered a novel DNA construct comprising a tissue-specific promoter and a nucleotide coding sequence which encodes an F5H enzyme. When heightened expression of F5H is achieved in a transformed plant in accordance with the present invention, the transformed plant accumulates lignin that is highly enriched in syringyl residues, and thereby is more readily degraded during the pulping process and during ruminant digestion of lignocellulosic feedstocks. As such, advantageous features of the present invention include the transformation of a wide variety of plants of various agriculturally and/or commercially valuable plant species to provide transformed plants having advantageous delignification properties. It is also expected that inventive tissue-specific promoters may be used in conjuction with expression, antisense or cosupression systems corresponding to other enzymes of the phenylpropanoid pathway, such as, for example, CAD or OMT, to enhance the effect of these systems in lignin-producing cells. While these systems have proven to have certain effects when present in a construct under the control of, for example, the 35S promoter, it is expected that placing the nucleotide sequence under control of a promoter selected in accordance with the present invention will enhance the desired result achieved using expression systems known in the prior art.

Promoters selected for use in accordance with one aspect of the present invention effectively

target F5H expression to those tissues that undergo lignification. Preferably, the promoter is one isolated from a gene which encodes an enzyme in the phenylpropanoid pathway. For example, over-expression of F5H may preferably be obtained in target plant tissues using one of the following promoters: phenylalanine ammonia-lyase (PAL), C4H, O-methyltransferase (OMT), (hydroxy)cinnamoyl-CoA ligase (4CL), (hydroxy)cinnamoyl-CoA reductase (CCR), (hydroxy)cinnamoyl alcohol dehydrogenase (CAD), Laccase and caffeic acid/ 5-hydroxyferulic acid. Most preferably, the promoter used is the C4H promoter. It is not intended, however, that this list be limiting, but only provide examples of promoters which may be advantageously used in accordance with the present invention to provide over-expression of F5H in cells producing lignin or providing precursors for lignin biosynthesis. Although promoter sequences for specific enzymes commonly differ between species, it is understood that the present invention includes promoters which regulate phenylpropanoid genes in a wide variety of plant species. For example, while the C4H promoter of the species *Arabidopsis thaliana* is set forth in SEQ ID NO:1 herein, it is not intended that the present invention be limited to this sequence, but include sequences having substantial similarity thereto and sequences from different plant species which promote the expression of analogous enzymes of that species' phenylpropanoid pathway.

Similarly, an expression sequence selected for use in accordance with the present invention is one that effectively modifies lignin biosynthesis in tissues that undergo lignification. Preferably, the expression sequence encodes an enzyme in the phenylpropanoid pathway. For example, over-expression, antisense, or cosuppression of lignin biosynthetic genes may preferably be obtained in the target plant tissues using an expression sequence encoding one of the following enzymes: PAL, C4H, OMT, F5H, 4CL, CCR, CAD, and laccase. Most preferably, the sequence used encodes F5H. It is not intended, however, that this list be limiting, but only provide examples of sequences which may be advantageously used in accordance with the present invention to provide over-expression, antisense or cosuppression of lignin biosynthetic enzymes in cells producing lignin or providing precursors for lignin biosynthesis. As sequences encoding related enzymes commonly differ between species, it is understood that the present invention includes genes which encode lignin

biosynthetic proteins in a wide variety of plant species. While nucleotide sequences encoding the F5H of the species *Arabidopsis thaliana* are set forth in SEQ ID NO:2 and SEQ ID NO:3 herein, it is not intended that the present invention be limited to these sequences, but include sequences having substantial similarity thereto and sequences from different plant species that encode enzymes involved in lignin biosynthesis of that species' phenylpropanoid pathway.

While the present invention is intended to encompass constructs comprising a wide variety of promoters and a wide variety of expressible nucleotide sequences, for purposes of describing the invention, one particularly preferred construct will be described as a representative example. It should be understood that this discussion applies equally to constructs prepared or selected in accordance with the invention which comprise a different promoter and/or a different coding sequence. The example described below comprises a C4H promoter and an F5H expression sequence. In this regard, nucleotide sequences advantageously selected for inclusion in a DNA construct according to a preferred aspect of the invention are a C4H regulatory sequence (as set forth in the C4H genomic sequence of SEQ ID NO:1) and either an F5H genomic sequence (as set forth in SEQ ID NO:2) or an F5H cDNA sequence (as set forth in SEQ ID NO:3).

The term "nucleotide sequence" is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, and derivatives thereof. The terms "encoding" and "coding" refer to the process by which a gene, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a functional protein, such as, for example, an active enzyme. It is understood that the process of encoding a specific amino acid sequence may involve DNA sequences having one or more base changes (i.e., insertions, deletions, substitutions) that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence.

A preferred DNA construct selected or prepared in accordance with the invention expresses an F5H enzyme, or an enzyme having substantial similarity thereto and having a level of enzymatic

activity suitable to achieve the advantageous result of the invention. A preferred amino acid sequence encoded by an inventive DNA construct is the F5H amino acid sequence set forth in SEQ ID NO:4. The terms "protein. "amino acid sequence" and "enzyme" are used interchangeably herein to designate a plurality of amino acids linked in a serial array. Skilled artisans will recognize that through the process of mutation and/or evolution. proteins of different lengths and having differing constituents, e.g., with amino acid insertions. substitutions. deletions. and the like. may arise that are related to the proteins of the present invention by virtue of (a) amino acid sequence homology; and (b) good functionality with respect to enzymatic activity. For example. an F5H enzyme isolated from one species and/or the nucleotide sequence encoding it. may differ to a certain degree from the sequences set forth herein. and yet have excellent functionality in accordance with the invention. Such an enzyme and/or nucleotide sequence falls directly within the scope of the present invention. While many deletions, insertions. and. especially. substitutions. are not expected to produce radical changes in the characteristics of the protein. when it is difficult to predict the exact effect of the substitution. deletion. or insertion in advance of doing so. one skilled in the art will appreciate that the effect may be evaluated by routine screening assays.

In addition to the F5H protein in this embodiment. therefore, the present invention also contemplates proteins having substantial identity thereto. The term "substantial identity." as used herein with respect to an amino acid sequence. is intended to mean sufficiently similar to have suitable functionality when expressed in a plant transformed in accordance with the invention to achieve the advantageous result of the invention. In one preferred aspect of the present invention. variants having such potential modifications as those mentioned above, which have at least about 50% identity to the amino acid sequence set forth in SEQ ID NO:4. are considered to have "substantial identity" thereto. Sequences having lesser degrees of identity but comparable biological activity are considered to be equivalents. It is believed that the identity required to maintain proper functionality is related to maintenance of the tertiary structure of the protein such that specific interactive sequences will be properly located and will have the desired activity. As such. it is believed that there are discrete domains and motifs within the amino acid sequence which

must be present for the protein to retain its advantageous functionality and specificity. While it is not intended that the present invention be limited by any theory by which it achieves its advantageous result, it is contemplated that a protein including these discrete domains and motifs in proper spatial context will retain good enzymatic activity.

It is therefore understood that the invention also encompasses more than the specific exemplary nucleotide sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce "silent" changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alterations in the nucleotide sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product.

Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity in the encoded products. As a related matter, it is understood that similar base changes may be present in a promoter sequence without substantially affecting its valuable functionality. Such variations to a promoter sequence are also within the purview of the invention.

In a preferred aspect, therefore, the present invention contemplates nucleotide sequences having substantial identity to those set forth in SEQ ID NOS. 1, 2 and 3. The term "substantial identity" is used herein with respect to a nucleotide sequence to designate that the nucleotide

sequence has a sequence sufficiently similar to one of those explicitly set forth herein that it will hybridize therewith under moderately stringent conditions, this method of determining identity being well known in the art to which the invention pertains. Briefly, moderately stringent conditions are defined in Sambrook et al., Molecular Cloning: a Laboratory Manual, 2ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989) as including the use of a prewashing solution of 5 x SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization and washing conditions of about 55 C, 5 x SSC. A further requirement of the term "substantial identity" as it relates to an inventive nucleotide coding sequence in accordance with this embodiment is that it must encode a protein having substantially similar functionality to the F5H enzyme set forth in SEQ ID NO:4, i.e., one which is capable of effecting an increased syringyl content in a plant's lignin composition when over-expressed in the plant's tissues producing lignin or providing the precursors for lignin biosynthesis.

Suitable DNA sequences selected for use according to the invention may be obtained, for example, by cloning techniques using cDNA libraries corresponding to a wide variety of plant species, these techniques being well known in the relevant art, or may be made by chemical synthesis techniques which are also well known in the art. Suitable nucleotide sequences may be isolated from DNA libraries obtained from a wide variety of species by means of nucleic acid hybridization or PCR, using as hybridization probes or primers nucleotide sequences selected in accordance with the invention, such as those set forth in SEQ ID NOS: 1, 2 and 3; nucleotide sequences having substantial identity thereto; or portions thereof. In certain preferred aspects of the invention, nucleotide sequences from a wide variety of plant species may be isolated and/or amplified which encode F5H, or a protein having substantial identity thereto and having suitable activity with respect to increasing syringyl content of the plant's lignin. Nucleotide sequences may also be isolated and/or amplified from a wide variety of plant species which correspond to the C4H promoter, a nucleotide sequence having substantial functional or sequence similarity thereto or a nucleotide sequence having an analogous function in a wide variety of plant species. Nucleotide sequences specifically set forth herein or selected in accordance with the invention may be

advantageously used in a wide variety of plant species, including but not limited to the species from which it is isolated.

Inventive DNA sequences can be incorporated into the genomes of plant cells using conventional recombinant DNA technology, thereby making transformed plants capable of producing lignin having increased syringyl content. In this regard, the term "genome" as used herein is intended to refer to DNA which is present in the plant and which is heritable by progeny during propagation of the plant. As such, inventive transgenic plants may alternatively be produced by breeding a transgenic plant made according to the invention with a second plant or selfing an inventive transgenic plant to form an F1 or higher generation plant. Transformed plants and progeny thereof are all contemplated by the invention and are all intended to fall within the meaning of the term "transgenic plant."

Generally, transformation of a plant involves inserting a DNA sequence into an expression vector in proper orientation and correct reading frame. The vector contains the necessary elements for the transcription of the inserted protein-encoding sequences. A large number of vector systems known in the art can be advantageously used in accordance with the invention, such as plasmids, bacteriophage viruses or other modified viruses. Suitable vectors include, but are not limited to the following viral vectors: lambda vector system gt11, gt10, Charon 4, and plasmid vectors such as pBI121, pBR322, pACYC177, pACYC184, pAR series, pKK223-3, pUC8, pUC9, pUC18, pUC19, pLG339, pRK290, pKC37, pKC101, pCDNAII, and other similar systems. The DNA sequences are cloned into the vector using standard cloning procedures in the art, for example, as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1982), which is hereby incorporated by reference. The plasmid pBI121 is available from Clontech Laboratories, Palo Alto, California. It is understood that related techniques may be advantageously used according to the invention to transform microorganisms such as, for example, *Agrobacterium sp.*, yeast, *E.coli* and *Pseudomonas sp.*

In order to obtain satisfactory expression of a lignification-related gene such as the F5H nucleotide coding sequence in the proper plant tissues, a tissue-specific plant promoter selected in

accordance with the invention must be present in the expression vector. An expression vector according to the invention may be either naturally or artificially produced from parts derived from heterologous sources, which parts may be naturally occurring or chemically synthesized, and wherein the parts have been joined by ligation or other means known in the art. The introduced coding sequence is under control of the promoter and thus will be generally downstream from the promoter. Stated alternatively, the promoter sequence will be generally upstream (i.e., at the 5' end) of the coding sequence. The phrase "under control of" contemplates the presence of such other elements as may be necessary to achieve transcription of the introduced sequence. As such, in one representative example, enhanced F5H production may be achieved by inserting a F5H nucleotide sequence in a vector downstream from and operably linked to a promoter sequence capable of driving tissue-specific high-level expression in a host cell. Two DNA sequences (such as a promoter region sequence and a F5H-encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired F5H-encoding gene sequence, or (3) interfere with the ability of the desired F5H sequence to be transcribed by the promoter region sequence.

RNA polymerase normally binds to the promoter and initiates transcription of a DNA sequence or a group of linked DNA sequences and regulatory elements (operon). A transgene, such as a nucleotide sequence selected in accordance with the present invention, is expressed in a transformed plant to produce in the cell a protein encoded thereby. Briefly, transcription of the DNA sequence is initiated by the binding of RNA polymerase to the DNA sequence's promoter region. During transcription, movement of the RNA polymerase along the DNA sequence forms messenger RNA ("mRNA") and, as a result, the DNA sequence is transcribed into a corresponding mRNA. This mRNA then moves to the ribosomes of the cytoplasm or rough endoplasmic reticulum which, with transfer RNA ("tRNA"), translates the mRNA into the protein encoded thereby. Proteins of the present invention thus produced in a transformed host then perform an important function in the plant's synthesis of lignin.

It is well known that there may or may not be other regulatory elements (e.g., enhancer sequences) which cooperate with the promoter and a transcriptional start site to achieve transcription of the introduced (i.e., foreign) coding sequence. Also, the recombinant DNA will preferably include a transcriptional termination sequence downstream from the introduced sequence.

Once the DNA construct of the present invention has been cloned into an expression system, it is ready to be transformed into a host plant cell. Plant tissue suitable for transformation in accordance with certain preferred aspects of the invention include whole plants, leaf tissues, flower buds, root tissues, meristems, protoplasts, hypocotyls and cotyledons. It is understood, however, that this list is not intended to be limiting, but only provide examples of tissues which may be advantageously transformed in accordance with the present invention. One technique of transforming plants with a DNA construct in accordance with the present invention is by contacting the tissue of such plants with an inoculum of a bacteria transformed with a vector comprising a DNA sequence selected in accordance with the present invention. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and incubating the tissue for about 48 to about 72 hours on regeneration medium without antibiotics at about 25-28 C.

Bacteria from the genus *Agrobacterium* may be advantageously utilized to transform plant cells. Suitable species of such bacterium include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its well-known ability to transform plants. Another technique which may advantageously be used is vacuum-infiltration of flower buds using *Agrobacterium*-based vectors.

Another approach to transforming plant cells with a DNA sequence selected in accordance with the present invention involves propelling inert or biologically active particles at plant tissues or cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006 and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles

are utilized. the vector can be introduced into the cell by coating the particles with the vector. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA material sought to be introduced) can also be propelled into plant cells. It is not intended, however, that the present invention be limited by the choice of vector or host cell. It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among vectors, expression control sequences, and hosts without undue experimentation and without departing from the scope of this invention.

Once the recombinant DNA is introduced into the plant tissue, successful transformants can be screened using standard techniques such as the use of marker genes, e.g., genes encoding resistance to antibiotics. Additionally, the level of expression of the foreign DNA may be measured at the transcriptional level, as protein synthesized or by assaying to determine lignin syringyl content.

An isolated DNA construct selected in accordance with the present invention may be utilized in an expression system to increase the syringyl content of lignin in a wide variety of plants, including gymnosperms, monocots and dicots. Inventive DNA constructs are particularly useful in the following plants: alfalfa *(Medicago* sp.), rice *(Oryza* sp.), maize *(Zea mays)*, oil seed rape *(Brassica* sp.), forage grasses, and also tree crops such as eucalyptus *(Eucalyptus* sp.), pine *(Pinus* sp.), spruce *(Picea* sp.) and poplar *(Populus* sp.), as well as *Arabidopsis* sp. and tobacco *(Nicotiana* sp.).

Those skilled in the art will recognize the commercial and agricultural advantages inherent in plants transformed to have increased or selectively increased expression of F5H and/or of nucleotide sequences which encode proteins having substantial identity thereto. Such plants are expected to have substantially improved delignification properties and, therefore, are expected to be more readily pulped and/or digested compared to a corresponding non-transformed plant.

The invention will be further described with reference to the following specific Examples. It will be understood that these Examples are illustrative and not restrictive in nature.

EXAMPLES

GENERAL METHODS

Restriction enzyme digestions, phosphorylations, ligations and transformations were done as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press. All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), " L" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "g" means gram(s), "mg" means milligram(s), " g" means microgram(s), "nm" means nanometer(s), "m" means meter(s), "E" means Einstein(s).

Plant material

Arabidopsis thaliana was grown under a 16 h light/8 h dark photoperiod at 100 E m^{-2} s^{-1} at 24 °C cultivated in Metromix 2000 potting mixture (Scotts, Marysville OH). Mutant lines *fah1-1* through *fah1-5* were identified by TLC as described below. Using their red fluorescence under UV light as a marker, mutant lines *fah1-6*, *fah1-7*, and *fah1-8* were selected from ethylmethane sulfonate (*fah1-6*, *fah1-7*) or fast neutron (*fah1-8*) mutagenized populations of Landsberg *erecta* M2 seed. The T-DNA tagged line 3590 (*fah1-9*) was similarly identified in the DuPont T-DNA tagged population (Feldmann, K.A., Malmberg, R.L., & Dean, C., (1994) Mutageneses in *Arabidopsis* in *Arabidopsis*, (E.M. Meyerowitz and C. R. Somerville, eds.) Cold Spring Harbor Press). All lines were backcrossed to wild type at least twice prior to experimental use to remove unlinked background mutations. Tobacco plants were grown in a greenhouse under a 16 h light/8 h dark photoperiod at 500 E m^{-2} s^{-1} at 24 °C cultivated in Metromix 2000 potting mixture (Scotts, Marysville OH).

Secondary Metabolite Analysis

Leaf extracts were prepared from 100 mg samples of fresh leaf tissue suspended in 1 mL of 50% methanol. Samples were vortexed briefly, then frozen at –70 °C. Samples were thawed, vortexed, and centrifuged at 12,000 xg for 5 min. Sinapoylmalate content was qualitatively determined following silica gel TLC, in a mobile phase of n-butanol/ethanol/water (4:1:1). Sinapic acid and its esters were visualized under long wave UV light (365 nm) by their characteristic fluorescence.

Southern Analysis

For Southern analysis, DNA was extracted from leaf material (Rogers, et al., (1985) *Plant. Mol. Biol.* 5, 69), digested with restriction endonucleases and transferred to Hybond N+ membrane (Amersham, Cleveland Ohio) by standard protocols. cDNA probes were radiolabelled with ^{32}P and hybridized to the target membrane in Denhardt's hybridization buffer (900 mM sodium chloride, 6 mM disodium EDTA, 60 mM sodium phosphate pH 7.4, 0.5% SDS, 0.01% denatured herring sperm DNA and 0.1% each polyvinylpyrrolidone, bovine serum albumin, and Ficoll 400) containing 50% formamide at 42 °C. To remove unbound probe, membranes were washed twice at room temperature and twice at 65 °C in 2x SSPE (300 mM sodium chloride, 2mM disodium EDTA, 20 mM sodium phosphate, pH 7.4) containing 0.1% SDS, and exposed to film.

Northern Analysis

RNA was first extracted from leaf material according to the following protocol. For extraction of RNA, Covey's extraction buffer was prepared by dissolving 1% (w/v) TIPS (triisopropyl-naphthalene sulfonate, sodium salt), 6% (w/v) PAS (*p*-aminosalicylate, sodium salt) in 50 mM Tris pH 8.4 containing 5% v/v Kirby's phenol. Kirby's phenol was prepared by neutralizing liquified phenol containing 0.1% (w/v) 8-hydroxyquinoline with 0.1 M Tris-HCl pH 8.8. For each RNA preparation, a 1 g samples of plant tissue was ground in liquid nitrogen and extracted in 5 mL Covey's extraction buffer containing 10 L -mercaptoethanol. The sample was extracted with 5 mL of a 1:1 mixture of Kirby's phenol and chloroform, vortexed, and centrifuged for 20 min at 7,000 xg. The supernatant was removed and the nucleic acids were precipitated with

500 L of 3 M sodium acetate and 5 mL isopropanol and collected by centrifugation at 10.000 xg for 10 min. The pellet was redissolved in 500 L water. and the RNA was precipitated on ice with 250 L 8 M LiCl. and collected by centrifugation at 10.000 xg for 10 min. The pellet was resuspended in 200 L water and extracted with an equal volume of chloroform:isoamyl alcohol 24:1 with vortexing. After centrifugation for 2 min at 10.000 xg. the upper aqueous phase was removed. and the nucleic acids were precipitated at –20 °C by the addition of 20 L 3 M sodium acetate and 200 L isopropanol. The pellet was washed with 1 mL cold 70% ethanol. dried. and resuspended in 100 L water. RNA content was assayed spectrophotometrically at 260 nm. Samples containing 1 to 10 g of RNA were subjected to denaturing gel electrophoresis as described elsewhere (Sambrook et al.. *supra*).

Extracted RNA was transferred to Hybond N+ membrane (Amersham. Cleveland Ohio). and probed with radiolabelled probes prepared from cDNA clones. Blots were hybridized overnight. washed twice at room temperature and once at 65 °C in 3x SSC (450 mM sodium chloride. 45 mM sodium citrate. pH 7.0) containing 0.1% SDS. and exposed to film.

Identification of cDNA and Genomic Clones

cDNA and genomic clones for F5H were identified by standard techniques using a 2.3 kb *Sac*II/*Eco*RI fragment from the rescued plasmid (pCC1) (Example 2) as a probe. The cDNA clone pCC30 was identified in the PRL2 library (Newman et al.. Plant Physiol. 106. 1241. (1994)) kindly provided by Dr. Thomas Newman (DOE Plant Research Laboratory. Michigan State University, East Lansing, MI). A genomic cosmid library of *Arabidopsis thaliana* (ecotype Landsberg *erecta*) generated in the binary cosmid vector pBIC 20 (Example 3) (Meyer et al.. *Science* 264. 1452. (1994) was screened with the radiolabelled cDNA insert derived from pCC30. Genomic inserts in the pBIC20 T-DNA are flanked by the neomycin phosphotransferase gene for kanamycin selection adjacent to the T-DNA right border sequence. and the -glucuronidase gene for histochemical selection adjacent to the left border. Positive clones were characterized by restriction digestion and Southern analysis in comparison to Arabidopsis genomic DNA.

Plant transformation

Transformation of *Arabidopsis thaliana* was performed by vacuum infiltration (Bent et al.. Science 265. 1856. (1994) with minor modifications. Briefly. 500 mL cultures of transformed *Agrobacterium* harboring the pBIC20-F5H cosmid. the pGA482-35S-F5H construct. or the pGA482 C4H-F5H construct were grown to stationary phase in Luria broth containing 10 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin. Cells were harvested by centrifugation and resuspended in 1 L infiltration media containing 2.2 g MS salts (Murashige and Skoog, *Physiol. Plant.* 15. 473. (1962)), Gamborg's B5 vitamins (Gamborg et al., *Exp. Cell Res.* 50. 151. (1968)). 0.5 g MES. 50 g sucrose. 44 nM benzylaminopurine. and 200 L Silwet L-77 (OSI Specialties) at pH 5.7. Bolting *Arabidopsis* plants (T_0 generation) that were 5 to 10 cm tall were inverted into the bacterial suspension and exposed to a vacuum (>500 mm of Hg) for three to five min. Infiltrated plants were returned to standard growth conditions for seed production. Transformed seedlings (T_1) were identified by selection on MS medium containing 50 mg L^{-1} kanamycin and 200 mg L^{-1} timentin (SmithKline Beecham) and were transferred to soil.

Transformation of tobacco was accomplished using the leaf disk method of Horsch et al. (*Science* 227, 1229, (1985)).

Nitrobenzene oxidation

For the determination of lignin monomer composition. stem tissue was ground to a powder in liquid nitrogen and extracted with 20 mL of 0.1 M sodium phosphate buffer. pH 7.2 at 37 °C for 30 min followed by three extractions with 80% ethanol at 80 °C. The tissue was then extracted once with acetone and completely dried. Tissue was saponified by treatment with 1.0 M NaOH at 37 °C for 24 hours. washed three times with water. once with 80% ethanol. once with acetone. and dried. Nitrobenzene oxidation of stem tissue samples was performed with a protocol modified from Iiyama et al. (*J. Sci. Food Agric.* 51. 481-491. (1990)). Samples of lignocellulosic material (5 mg each) were mixed with 500 L of 2 M NaOH and 25 L of nitrobenzene. This mixture was incubated in a sealed glass tube at 160 °C for 3 h. The reaction products were cooled to room temperature and 5 L of a 20 mg mL^{-1} solution of 3-ethoxy-4-hydroxybenzaldehyde in pyridine

was added as an internal standard before the mixture was extracted twice with 1 mL of dichloromethane. The aqueous phase was acidified with HCl (pH 2) and extracted twice with 900 L of ether. The combined ether phases were dried with anhydrous sodium sulfate and the ether was evaporated in a stream of nitrogen. The dried residue was resuspended in 50 L of pyridine. 10 L of BSA (N,O-bis-(trimethylsilyl)-trifluoracetamide) was added and 1 L aliquots of the silylated products were analyzed using a Hewlet-Packard 5890 Series II gas chromatograph equipped with Supelco SPB I column (30 m x 0.75 mm). Lignin monomer composition was calculated from the integrated areas of the peaks representing the trimethylsilylated derivatives of vanillin, syringaldehyde, vanillic acid and syringic acid. Total nitrobenzene oxidation-susceptible guaiacyl units (vanillin and vanillic acid) and syringyl units (syringaldehyde and syringic acid) were calculated following correction for recovery efficiencies of each of the products during the extraction procedure relative to the internal standard.

EXAMPLE ONE

Identification of the T-DNA Tagged Allele of FAH1

A putatively T-DNA tagged *fah1* mutant was identified in a collection of T-DNA tagged lines (Feldman et al., *Mol. Gen. Genet.* 208, 1, (1987)) (Dr. Tim Caspar, Dupont, Wilmington, DE) by screening adult plants under long wave UV light. A red fluorescent line (line 3590) was selected, and its progeny were assayed for sinapoylmalate content by TLC. The analyses indicated that line 3590 did not accumulate sinapoylmalate. Reciprocal crosses of line 3590 to a *fah1l-2* homozygote, followed by analysis of the F1 generation for sinapoylmalate content demonstrated that line 3590 was a new allele of *fah1*, and it was designated *fah1-9*.

Preliminary experiments indicated co-segregation of the kanamycin-resistant phenotype of the T-DNA tagged mutant with the *fah1* phenotype. Selfed seed from 7 kanamycin-resistant *[fah1-9 x FAH1]* F1 plants segregated 1:3 for kanamycin resistance (kan^{sensitive}: kan^{resistant}) and 3:1 for sinapoylmalate deficiency *(Fah1:fah1)*. From these lines, *fah1* plants gave rise to only kan^{resistant}, *fah1* progeny. To determine the genetic distance between the T-DNA insertion and

the FAH1 locus, multiple test crosses were performed between a *[fah1-9 x FAH1]* F1 and a *fah1-2* homozygote. The distance between the FAH1 locus and the T-DNA insertion was evaluated by determining the frequency at which *FAH1/kan*^{resistant} progeny were recovered in the test cross F1. In the absence of crossover events, all kanamycin-resistant F1 progeny would be unable to accumulate sinapoylmalate, and would thus fluoresce red under UV light. In 682 kan^{resistant} F1 progeny examined, no sinapoylmalate proficient plants were identified, indicating a very tight linkage between the T-DNA insertion site and the FAH1 locus.

EXAMPLE TWO
Plasmid Rescue and cDNA Cloning of the FAH1 Gene

Plasmid rescue was conducted using *Eco*RI-digested DNA prepared from homozygous *fah1*-9 plants (Behringer et al., Plant Mol. Biol. Rep. 10, 190, (1992)). Five g of *Eco*RI-digested genomic DNA was incubated with 125 U T4 DNA ligase overnight at 14 °C in a final volume of 1 mL. The ligation mixture was concentrated approximately four fold by two extractions with equal volumes of 2-butanol, and was then ethanol precipitated and electroporated into competent DH5- cells as described (Behringer et al., (1992) *supra*).

DNA from rescued plasmids was double digested with *Eco*RI and *Sal*I. Plasmids generated from internal T-DNA sequences were identified by the presence of triplet bands at 3.8, 2.4 and 1.2 kb and were discarded. One plasmid (pCC1) giving rise to the expected 3.8 kb band plus a novel 5.6 kb band was identified as putative external right border plasmid. Using a *Sac*II/*Eco*RI fragment of pCC1 that appeared to represent *Arabidopsis* DNA, putative cDNA (pCC30) clones for F5H were identified. The putative F5H clone carried a 1.9 kb *Sal*I-NotI insert, the sequence of which was determined. Blastx analysis (Altschul et al., *J. Mol. Biol.* 215, 403, (1990)) indicated that this cDNA encodes a cytochrome P450-dependent monooxygenase, consistent with earlier reports that (I) the *fah1* mutant is defective in F5H (Chapple et al., *supra.*) and (ii) F5H is a cytochrome P450-dependent monooxygenase (Grand, *supra*).

Southern and Northern Blot analysis

To determine whether the putative F5H cDNA actually represented the gene that was disrupted in the T-DNA tagged line Southern and northern analysis was used to characterize the available *fah1* mutants using the putative F5H cDNA.

Figure 2 shows a Southern blot comparing hybridization of the F5H cDNA to *Eco*RI-digested genomic DNA isolated from wild type (ecotypes Columbia (Col). Landsberg *erecta* (LER). and Wassilewskija (WS)) and the nine *fah1* alleles including the T-DNA tagged *fah1-9* allele. WS is the ecotype from which the T-DNA tagged line was generated.

These data indicated the presence of a restriction fragment length polymorphism between the tagged line and the wild type. These data also indicates a restriction fragment length polymorphism in the *fah1-8* allele which was generated with fast neutrons. a technique reported to cause deletion mutations.

As shown in Figure 2. the genomic DNA of the *fah1-8* and *fah1-9* (the T-DNA tagged line) alleles is disrupted in the region corresponding to the putative F5H cDNA. These date also indicate that F5H is encoded by a single gene in *Arabidopsis* as expected considering that the mutation in the *fah1* mutant segregates as a single Mendelian gene. These data provide the first indication that the putative F5H cDNA corresponds to the gene that is disrupted in the *fah1* mutants.

Plant material homozygous for nine independently-derived *fah1* alleles was surveyed for the abundance of transcript corresponding to the putative F5H cDNA using Northern blot analysis. The data is shown in Figure 3.

As can be seen from the data. the putative F5H mRNA was represented at similar levels in leaf tissue of Columbia. Landsberg *erecta* and Wassilewskija ecotypes. and in the EMS-induced *fah1-1*. *fah1-4*. and *fah1-5*. as well as the fast neutron-induced *fah1-7*. Transcript abundance was substantially reduced in leaves from plants homozygous for the *fah1-2*. *fah1-3* and *fah1-6*. all of which were EMS-induced. the fast neutron-induced mutant *fah1-8* and in the tagged line *fah1-9*. The mRNA a *fah1-8* mutant also appears to be truncated. These data provided strong evidence that the cDNA clone that had been identified is encoded by the FAH1 locus.

EXAMPLE THREE

Demonstration of the Identity of the F5H cDNA
by Transformation of *fah1* Mutant Plants With Wildtype F5H
and Restoration of Sinapoylmalate Accumulation

In order to demonstrate the identity of the F5H gene at the functional level, the transformation-competent pBIC20 cosmid library (Meyer et al., *supra*) was screened for corresponding genomic clones using the full length F5H cDNA as a probe. A clone (pBIC20-F5H) carrying a genomic insert of 17 kb that contains 2.2 kb of sequence upstream of the putative F5H start codon and 12.5 kb of sequence downstream of the stop codon of the F5H gene (Figure 4) was transformed into the *fah1-2* mutant by vacuum infiltration. Thirty independent infiltration experiments were performed, and 167 kanamycin-resistant seedlings, representing at least 3 transformants from each infiltration, were transferred to soil and were analyzed with respect to sinapic acid-derived secondary metabolites. Of these plants, 164 accumulated sinapoylmalate in their leaf tissue as determined by TLC (Figure 5). These complementation data indicate that the gene defective in the *fah1* mutant is present on the binary cosmid pBIC20-F5H.

To delimit the region of DNA on the pBIC20-F5H cosmid responsible for complementation of the mutant phenotype, a 2.7 kB fragment of the F5H genomic sequence was fused downstream of the cauliflower mosaic virus 35S promoter in the binary plasmid pGA482 and this construct (pGA482-35S-F5H) (Figure 4) was transformed into the *fah1* mutant. The presence of sinapoyl malate in 109 of 110 transgenic lines analyzed by TLC or by *in vivo* fluorescence under UV light indicated that the *fah1* mutant phenotype had been complemented (Figure 5). These data provide conclusive evidence that the F5H cDNA has been identified.

EXAMPLE FOUR

DNA Sequencing of the F5H cDNA
and Genomic Clones

The F5H cDNA and a 5156 bp *Hind*III-XhoI fragment of the pBIC20-F5H genomic clone were both fully sequenced on both strands and the sequence of the F5H protein (SEQ ID NO.:4) was inferred from the cDNA sequence. The sequence of the *Arabidopsis thaliana* F5H cDNA is given in SEQ ID NO.:2. The sequence of the *Arabidopsis thaliana* F5H genomic clone is given in SEQ ID NO.:3.

EXAMPLE FIVE

Identification and DNA Sequencing of the C4H Promoter Sequence

A search of the Arabidopsis EST library using the keyword "cinnamate" identified a number of clones, most of which corresponded to members of the cytochrome P450 gene superfamily. One of these sequences (clone ID# 126E1T7, Genbank accession number T44874) was highly homologous to C4H sequences characterized from mung bean and Jerusalem artichoke (Mizutani et al., Biochem. Biophys. Res. Commun. 190, 875, (1993); Teutsch et al., Proc. Natl. Acad. Sci. USA 90, 4102, (1993)). This clone also appeared to be a full length P450 cDNA, thus the C4H cDNA EST clone 126E1T7 was obtained from the Ohio State Arabidopsis Resource Center. The putative C4H cDNA was sequenced and was found to be 69 to 72% identical to C4H sequences available in the database and its deduced amino acid sequence shares 84 to 86% identity. To evaluate whether C4H is encoded at a single locus in Arabidopsis, the C4H cDNA was used as a probe against Arabidopsis DNA digested with a number of restriction enzymes (Figure 6). The probe hybridized to a single band in all lanes except those containing the *Xma*I and *Sty*I digests, consistent with the presence of sites for these enzymes within the cDNA. Comparison of the hybridization banding pattern obtained with Columbia and Landsberg *erecta* DNA identified a restriction fragment length polymorphism with *Sty*I. This polymorphism permitted the mapping of the C4H

gene to the lower arm of chromosome 2 using recombinant inbred populations (Lister and Dean, Plant J., 4, 745, (1993)). The C4H locus maps to a position 0.8 cM below the marker m283c and 5.1 cM above the marker m323. Further evidence that C4H is encoded by a single gene in Arabidopsis was provided by searching the *Arabidopsis thaliana* EST database with the full length C4H cDNA sequence. This search retrieved the EST whose sequence is reported here as well as four other sequences (Genbank accession numbers F19837, T04086, N65601, T43776) that are essentially identical to the full length C4H cDNA sequence. The similarity of the C4H cDNA sequence to all others in the database is substantially less after these five are considered. This suggests that there are no other closely related C4H-like genes expressed in Arabidopsis.

Using the C4H cDNA as a probe, a genomic cosmid library was screened to identify a C4H genomic clone from a Landsberg *erecta* genomic library generated in the binary cosmid vector pBIC20 (Meyer et al., *supra*). Twelve overlapping genomic clones were isolated that covered the C4H locus, and restriction analysis revealed that these clones fell into three different classes. Southern blot analysis indicated each clone contained a *Hin*dIII fragment that carried the entire C4H coding sequence. This 5.4 kb *Hin*dIII DNA fragment containing the entire C4H coding sequence from one of the cosmids was subcloned into pGEM-7Zf(+) (Promega) in both the 5'-3' and 3'-5' orientation and transformed into *E. coli* DH5 . Alignment of the genomic sequence with the cDNA revealed that the subcloned fragment carried approximately 3 kb of upstream regulatory sequence and that the C4H coding sequence is interrupted by two small introns (intron I, 85 bp; intron II, 220 bp). The sequence of the *Arabidopsis thaliana* C4H genomic DNA is given in SEQ ID NO.:1.

The transcription start site of the C4H gene was determined by primer extension using an oligonucleotide (5'-CCATTATAGTTTGTGTATCCGC-3') complementary to the 5' end of the C4H cDNA clone. This oligonucleotide was end-labeled with [-^{32}P]ATP using polynucleotide kinase, and an amount of labeled primer equaling 400,000 cpm was added to 20 g of total RNA isolated from Arabidopsis stems, precipitated and dried. The DNA-RNA hybrids were dissolved in 30 L of hybridization buffer (80% formamide, 1 mM EDTA, 0.4 M NaCl, 14 mM PIPES, pH 6.4).

incubated at 85 C for 10 min and at 28 C overnight, and reprecipitated. The dried pellet was resuspended in 20 L of reverse transcriptase buffer, and the primer was extended using Moloney murine leukemia virus reverse transcriptase (Gibco). The extended product was analyzed by gel electrophoresis adjacent to the products of a sequencing reaction performed with the primer extension oligonucleotide and the C4H genomic clone. The transcription start site for the C4H mRNA was determined to be 86 bp upstream of the initiator ATG. A putative TATA box is found 33 bp upstream of the transcription start site, and a putative CAAT box at -152.

A C4H-GUS transcriptional fusion was constructed using a 2897 bp C4H promoter nested deletion clone carrying the C4H transcription start site. The 3' end of the selected clone terminated at position +34 within the region corresponding to the 5' untranslated region of the C4H cDNA. This fragment was liberated from pGEM-7Zf(+) by digestion with *Hin*dIII and *Apa*I and was subcloned into *Hin*dIII-*Sma*I-digested pBI101 using an *Apa*I-blunt-ended adaptor. Ligation products were transformed into *E. coli* NM544. The recombinant plasmids were characterized by diagnostic restriction digests prior to use in plant transformation experiments. To evaluate the tissue specificity of C4H promoter-driven GUS expression in transgenic plants, tissues from kanamycin-resistant T_1 Arabidopsis plants were incubated in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl- -D-glucuronide (X-Gluc), 100 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100 from 8 to 12 hours at 37 C (Stomp, 1992). Tissues were destained three times in 70% ethanol and whole mounts and sections were analyzed by bright field microscopy.

Among a large number of T1 transformant seedlings carrying the C4H-GUS transcriptional fusion, GUS staining patterns were observed (Figure 7) that were consistent with RNA blot data obtained using the C4H cDNA probe. In cotyledons, GUS staining was diffusely distributed throughout the epidermis and mesophyll with higher levels of staining localized to the vascular tissue and the surrounding parenchyma (Figure 7). Strong staining was also seen in structures at the cotyledonary margins that resemble hydathodes. In the meristematic region of the seedling, strong GUS activity was present in the developing primary leaves where staining was diffusely distributed.

and was not localized to the developing vascular tissue. The highest level of GUS staining in the seedling was observed in the root. This high level of GUS staining was relatively clearly demarcated beginning at the hypocotyl/root junction, and continuing to near the root tip (Figure 7).

In mature leaves. GUS staining was very strongly localized to the veins (Figure 7). Similarly, expression of GUS activity in stem cross-sections was restricted to the xylem and the sclerified parenchyma that extends between the vascular bundles (Figure 7). In reproductive tissues, weak GUS staining was seen throughout the flower including the vasculature of the sepals, with stronger staining evident immediately below the stigmatic surface (Figure 7).

These data indicate that the Arabidopsis C4H gene has been identified, and that the region of DNA upstream of the C4H coding sequence defines a functional C4H promoter that is capable of directing gene expression in the vascular tissue of transgenic plants.

EXAMPLE SIX

Modification of Lignin Composition in Plants Transformed With F5H
Under the Control of the Cauliflower Mosaic Virus 35S Promoter

Arabidopsis plants homozygous for the *fah1-2* allele were transformed with *Agrobacterium* carrying the pGA482-35S-F5H plasmid which contains the chimeric F5H gene under the control of the constitutive cauliflower mosaic virus 35S promoter. Independent homozygous transformants carrying the F5H transgene at a single genetic locus were identified by selection on kanamycin-containing growth media, grown up in soil and plant tissue was analyzed for lignin monomer composition. Nitrobenzene oxidation analysis of the lignin in wild type, *fah1-2*, and transformants carrying the T-DNA from the pGA482-35S-F5H construct revealed that F5H over-expression as measured by northern blot analysis led to a significant increase in syringyl content of the transgenic lignin (Figure 8). The lignin of the F5H-over-expressing plants demonstrated a syringl content as high as 29 mol% as opposed to the syringyl content of the wild type lignin which was 18 mol% (Table 1, Figure 8). In addition, histochemical staining of rachis cross sections indicated that the tissue specificity of syringyl lignin deposition was abolished in transgenic lines ectopically expressing F5H (Figure 9). Syringyl unit deposition was no longer restricted to the cells of the

sclerified parenchyma but was also found in the lignin deposited by the cells of the vascular bundle. This indicates that cells of the vascular bundle are competent to synthesize, secrete and polymerize monolignols derived from sinapic acid if they are made competent to express an active F5H gene. These data clearly demonstrate that over-expression of the F5H gene is useful for the alteration of lignin composition in transgenic plants.

TABLE 1

Impact of 35S Promoter-Driven F5H Expression on Lignin Monomer Composition in *Arabidopsis*

Line		total G units[a] (μmol g^{-1} d.w.)	total S units[b] (μmol g^{-1} d.w.)	total G+S units (μmol g^{-1} d.w.)	mol % S
wild type		3.33 +/- 0.32	0.75 +/- 0.09	4.09 +/- 0.41	18.4 +/- 0.91
fah1-2		5.44 +/- 0.45	n.d.	5.44 +/- 0.45	-
88	(A)	6.63 +/- 0.75	0.35 +/- 0.04	6.99 +/- 0.79	5.06 +/- 0.17
172	(B)	4.21 +/- 0.36	0.67 +/- 0.07	4.88 +/- 0.42	13.7 +/- 0.55
170	(C)	4.08 +/- 0.33	0.97 +/- 0.06	5.05 +/- 0.37	19.2 +/- 0.56
122	(D)	3.74 +/- 0.20	0.93 +/- 0.05	4.66 +/- 0.22	19.9 +/- 0.86
108	(E)	5.40 +/- 0.48	1.59 +/- 0.18	6.98 +/- 0.65	22.7 +/- 0.82
107	(F)	5.74 +/- 0.60	1.96 +/- 0.31	7.70 +/- 0.89	25.3 +/- 1.23
180	(G)	3.85 +/- 0.31	1.34 +/- 0.11	5.19 +/- 0.40	25.8 +/- 0.78
117	(H)	3.21 +/- 0.30	1.18 +/- 0.13	4.39 +/- 0.43	28.8 +/- 0.92
128	(I)	3.46 +/- 0.22	1.39 +/- 0.17	5.05 +/- 0.37	27.5 +/- 1.80

[a] sum of vanillin + vanillic acid
[b] sum of syringaldehyde + syringic acid
n.d not detectable

In similar fashion, T1 tobacco (*Nicotiana tabacum*) pGA482 35S-F5H transformants were generated, grown up and analyzed for lignin monomer composition. Nitrobenzene oxidation analysis demonstrated that the syringyl monomer content of the leaf midribs was increased from 14 mol% in the wild type to 40 mol% in the transgenic line that most highly expressed the F5H transgene (Table 2). In contrast, nitrobenzene oxidation analysis of stem tissue demonstrated that in the syringyl lignin content of both wild type and the pGA482 35S-F5H transformants were both approximately 50% (Table 3). These data indicate that the overexpression of F5H directed by the 35S promoter is of limited efficacy in tissues that undergo secondary growth such as tobacco stem. Thus, the pGA482 35S-F5H can be expected to be of limited utility in the modification of lignin

monomer composition in trees.

TABLE 2

Impact of 35S Promoter-Driven F5H Expression on Lignin Monomer Composition in Tobacco Leaf Midrib Xylem

Line	total G units[a] (µmol g^{-1} d.w.)	total S units[b] (µmol g^{-1} d.w.)	total G+S units (µmol g^{-1} d.w.)	mol % S
wild type	1.40 +/- 0.26	0.23 +/- 0.04	1.63 +/- 0.30	14.3 +/- 1.09
40	0.86 +/- 0.16	0.24 +/- 0.03	1.11 +/- 0.20	22.4 +/- 1.53
27	1.13 +/- 0.11	0.52 +/- 0.05	1.65 +/- 0.16	31.3 +/- 0.50
48	1.28 +/- 0.32	0.71 +/- 0.19	1.99 +/- 0.43	35.7 +/- 6.06
33	0.65 +/- 0.17	0.43 +/- 0.11	1.09 +/- 0.27	40.0 +/- 1.86

[a] sum of vanillin + vanillic acid
[b] sum of syringaldehyde + syringic acid

TABLE 3

Impact of 35S Promoter-Driven F5H Expression on Lignin Monomer Composition in Tobacco Stem Xylem

Line	total G units[a] (µmol g^{-1} d.w.)	total S units[b] (µmol g^{-1} d.w.)	total G+S units (µmol g^{-1} d.w.)	mol % S
wild type	5.53 +/- 0.64	5.39 +/- 0.60	10.9 +/- 1.07	49.3 +/- 2.80
40	4.28 +/- 0.36	5.16 +/- 0.35	9.45 +/- 0.57	54.7 +/- 2.20
27	4.06 +/- 0.32	4.26 +/- 0.36	8.32 +/- 0.60	51.2 +/- 1.76
48	5.78 +/- 0.38	6.28 +/- 0.66	12.1 +/- 1.00	52.0 +/- 1.67
33	5.79 +/- 0.40	4.58 +/- 0.29	10.4 +/- 0.69	44.2 +/- 0.15

[a] sum of vanillin + vanillic acid
[b] sum of syringaldehyde + syringic acid

The data in Tables 1 and 2 clearly demonstrate that over-expression of the F5H gene in transgenic plants results in the modification of lignin monomer composition. The transformed plant is reasonably expected to have syringyl lignin monomer content that up to about 35 mol% as measured in whole plant tissue. The data in Table 3, however, indicate that the 35S promoter may be of limited efficacy in the modification of lignin biosynthesis in transgenic plants that undergo secondary growth, and in those plants whose syringyl lignin content naturally exceeds 35%.

EXAMPLE SEVEN

Modification of Lignin Composition in Plants Transformed With F5H Under the Control of the C4H Promoter

Given the limited efficacy of the pGA482 35S-F5H construct, a new construct was developed in which F5H transcription was driven by regulatory sequences of the C4H gene and this DNA construct was transformed into *fah1-2* mutant plants. Lignin analysis of transgenic rachis tissue revealed that expression of F5H under the control of the C4H promoter resulted in the production of a lignin with a syringyl content that greatly exceeded that observed in the 35S-F5H transgenics, despite the fact that the levels of F5H mRNA in these transgenic lines were substantially lower than those in the 35S-F5H transgenics (Table 4, Figures 10 and 11). In several of the transgenic lines, the lignin was almost solely comprised of syringyl residues. As in the 35S-F5H transgenics, the tissue specificity of syringyl lignin deposition was abolished in plants carrying the C4H-F5H transgene (Figure 9). When grown under the same controlled conditions, the C4H-F5H transgenic plants were phenotypically indistinguishable from wild type plants.

TABLE 4

Impact of C4H Promoter-Driven F5H Expression on Lignin Monomer Composition in *Arabidopsis*

Line		total G units[a] (μmol g^{-1} d.w.)	total S units[b] (μmol g^{-1} d.w.)	total G+S units (μmol g^{-1} d.w.)	mol % S
wild type		4.81 +/- 0.62	1.18 +/- 0.27	6.00 +/- 0.86	19.6 +/- 2.31
fah1-2		6.27 +/- 1.25	n.d.	6.27 +/- 0.45	-
1861	(J)	4.25 +/- 0.65	3.45 +/- 0.48	7.70 +/- 1.10	44.8 +/- 1.67
1786	(K)	3.97 +/- 0.72	3.59 +/- 0.60	7.56 +/- 1.31	47.5 +/- 0.96
1821	(L)	2.31 +/- 0.34	5.53 +/- 0.45	7.84 +/- 0.72	70.6 +/- 1.86
1794	(M)	1.46 +/- 0.18	5.05 +/- 0.34	6.51 +/- 0.43	77.6 +/- 2.03
1876	(N)	1.24 +/- 0.24	5.91 +/- 1.44	7.15 +/- 1.67	82.5 +/- 0.97
1875	(O)	1.30 +/- 0.10	7.49 +/- 0.68	8.79 +/- 0.76	85.2 +/- 0.76
1863	(P)	0.82 +/- 0.13	7.38 +/- 1.59	8.20 +/- 1.72	90.0 +/- 0.50
1844	(Q)	0.85 +/- 0.16	7.67 +/- 1.28	8.52 +/- 1.40	90.1 +/- 0.26
1824	(R)	0.53 +/- 0.07	6.15 +/- 0.93	6.67 +/- 0.99	92.1 +/- 0.42

[a] sum of vanillin + vanillic acid
[b] sum of syringaldehyde + syringic acid

Similar analyses of tobacco plants transformed with the pGA482 C4H-F5H construct demonstrated that expression of F5H under the control of the C4H promoter resulted in the production of a lignin with a syringyl content that greatly exceeded that observed in the 35S-F5H tobacco transgenics (Table 5). These data indicate that while the 35S-F5H construct leads to an increase in syringyl monomer content in the lignin of leaves. the construct has little utility in woody tissues such as tobacco stem. In contrast. the C4H-F5H overexpression construct shows a greater efficacy in tobacco stems. and thus provides the ability to modify the lignin monomer composition of other woody species. It should be noted that as in the case of the Arabidopsis C4H-F5H transgenics. the C4H-F5H transgenic plants were phenotypically indistinguishable from wild type plants.

TABLE 5

Impact of C4H Promoter-Driven F5H Expression on Lignin Monomer Composition in Tobacco Stem Xylem

Line	total G units[a] (μmol g^{-1} d.w.)	total S units[b] (μmol g^{-1} d.w.)	total G+S units (μmol g^{-1} d.w.)	mol % S
wild type	6.20 +/- 0.51	6.42 +/- 0.44	12.6 +/- 0.89	50.1 +/- 1.40
37	3.42 +/-1.15	3.04 +/- 1.20	6.28 +/- 2.34	48.1 +/- 1.67
2	4.38 +/- 0.77	7.68 +/- 1.46	12.1 +/- 2.17	63.7 +/- 1.99
32	2.24 +/- 0.37	5.77 +/- 1.16	8.01 +/- 0.71	71.9 +/- 1.35
9	3.08 +/- 0.34	11.2 +/- 1.61	14.3 +/- 1.87	78.4 +/- 1.64
8	2.28 +/- 0.40	8.84 +/- 1.78	11.1 +/- 2.18	79.4 +/- 0.57
18	2.45 +/- 0.17	9.68 +/- 1.82	12.1 +/- 1.98	79.6 +/- 1.91
35	1.52 +/- 0.17	8.16 +/- 1.22	9.69 +/- 1.38	84.2 +/- 0.76

[a] sum of vanillin + vanillic acid
[b] sum of syringaldehyde + syringic acid

These results demonstrate that the composition of the lignin polymer is dictated by the temporal and tissue-specific expression pattern of F5H in Arabidopsis and tobacco. It has further been shown that the CaMV 35S promoter. which frequently has been used in transgenic studies

aimed at the modification of lignin biosynthesis, fails to promote F5H gene expression in cells undergoing or providing precursors for lignification. The promoter of the C4H gene used in this study is far more efficient in this regard and will be a very valuable tool in transgenic studies addressing plant lignification in the future. These data also indicate that the use of other endogenous promoters in biotechnological applications may enhance not only tissue-specificity but also tissue-efficacy of transgene expression when compared to non-specific ectopic promoters such as the CaMV 35S promoter. Finally, it is shown herein that it is possible to genetically engineer plants to accumulate lignin that is highly enriched in syringyl residues. The unaltered morphology of tracheary elements and sclerified parenchyma in transgenic plants made in accordance with the invention suggests that this lignin still provides lignified cells with sufficient rigidity to function normally in water conduction and mechanical support.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Chapple, Clinton C. S.

(ii) TITLE OF INVENTION: MANIPULATIN OF LIGNIN COMPOSITION IN PLANTS USING A TISSUE-SPECIFIC PROMOTER

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.5:, 1.44Mb
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(C) OPERATING SYSTEM: MSDOS
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(2) INFORMATION FOR SEQ ID NO:1;

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5432 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAAGAGATTG GGAAGAGAGA AGAGACGATA AAGGAAACGG AAAAGCATAT GGAGGAGCTT   120
CATATGGAGC AAGTGAGGCT GAGAAGACGG TCGAGTGAGC TTACGGAAGA AGTGGAAAGG   180
ACGAGAGTGT CTGCATCGGA AATGGCTGAG CAGAAAAGAG AAGCTATAAG ACAGCTTTGT   240
ATGTCTCTTG ACCATTACAG AGATGGGTAC GACAGGCTTT GGAGAGTTGT TGCCGGCCAT   300
AAGAGTAAGA GAGTAGTGGT TTTAACAACT TGAAGTGTAA GAACAATGAG TCAATGACTA   360
CGTGCAGGAC ATTGGACATA CCGTGTGTTC TTTTGGATTG AAATGTTGTT TCGAAGGGCT   420
GTTAGTTGAT GTTGAAAATA GGTTGAAGTT GAATAATGCA TGTTGATATA GTAAATATCA   480
ATGGTAATAT TTTCTCATTT CCCAAAACTC AAATGATATC ATTTAATTAT AAACTAACGT   540
AAACTGTTGA CAATACACTT ATGGTTAAAA ATTTGGAGTC TTGTTTTAGT ATACGTATCA   600
CCACCGCACG GTTTCAAAAC CACATAATTG TAAATGTTAT TGGAAAAAAG AACCCGCAAT   660
ACGTATTGTA TTTTGGTAAA CATAGCTCTA AGCCTCTAAT ATATAAGCTC TCAACAATTC   720
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GGGTGAGGTG GAATACTAAC ATGAGGAGAA AGTAAGGTGA CAAATTTTTG GGGCAATAGT   840
GGTGGATATG GTGGGGAGGT AGGTAGCATC ATTTCTCCAA GTCGCTGTCT TTCGTGGTAA   900
TGGTAGGTGT GTCTCTCTTT ATATTATTTA TTACTACTCA TTGTTAATTT CTTTTTTTCT   960
ACAATTTGTT TCTTACTCCA AAATACGTCA CAAATATAAT ACTAGGCAAA TAATTATTTA  1020
ATTGTAAGTC AATAGAGTGG TTGTTGTAAA ATTGATTTTT GATATTGAAA GAGTTCATGG  1080
ACGGATGTGT ATGCGCCAAA TGCTAAGCCC TTGTAGTCTT GTACTGTGCC GCGCGTATAT  1140
TTTAACCACC ACTAGTTGTT TCTCTTTTTC AAAAACACAC AAAAAATAAT TTGTTTTCGT  1200
AACGGCGTCA AATCTGACGG CGTCTCAATA CGTTCAATTT TTTCTTTCTT TCACATGGTT  1260
TCTCATAGCT TTGCATTGAC CATAGGTAAA GGGATAAGGA TAAAGGTTTT TTCTCTTGTT  1320
TGTTTTATCC TTATTATTCA AAATGGATAA AAAAACAGTC TTATTTTGAT TTCTTTGATT  1380
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AAAAAAGTCA TTGAAATTCA TATTTGATTT TTTGCTAAAT GTCAACTCAG AGACACAAAC 1440
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AATCAGTAAA ATGCAAACAA AGCATTTTCT AATTAAAACA GTCTTCTACA TTCACTTAAT 1560
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AAAACTATTG GCGGGTTGGT CTATCCGAAT TGAAGATCTT TTCTCCATAT GATAGACCAA 1680
CGAAATTCGG CATACGTGTT TTTTTTTTTG TTTTGAAAAC CCTTTAAACA ACCTTAATTC 1740
AAAATACTAA TGTAACTTTA TTGAACGTGC ATCTAAAAAT TTTGAACTTT GCTTTTGAGA 1800
AATAATCAAT GTACCAATAA AGAAGATGTA GTACATACAT TATAATTAAA TACAAAAAAG 1860
GAATCACCAT ATAGTACATG GTAGACAATG AAAAACTTTA AAACATATAC AATCAATAAT 1920
ACTCTTTGTG CATAACTTTT TTTGTCGTCT CGAGTTTATA TTTGAGTACT TATACAAACT 1980
ATTAGATTAC AAACTGTGCT CAGATACATT AAGTTAATCT TATATACAAG AGCACTCGAG 2040
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TATTTTCTTT TTTTTACCAT GAGCAAAAAA AGATGAAGTA AGTTCAAAAC GTGACGAATC 2160
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GTACTACAAA ACTCCTAAGA GTGAGAACGA CTACATAGTA CATATTTTGA TAAAAGACTT 2280
GAAAACTTGC TAAAACGAAT TTGCGAAAAT ATAATCATAC AAGTAGAACC ACTGATTTGA 2340
TCGAATTATT CATAGCTTTG TAGGATGAAC TTAACTAAAT AATATCTCAC AAAAGTATTG 2400
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CACTTATTCA TATGATTTTT GAAGCAACTA CTTTCGTTTT TTTAACATTT TCTTTTTTGG 2520
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GAACTTTATT TAATAGATAT GAACATAATC TCACATCCTC CTCCTACCTT CACCAAACAC 2640
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TCCCACGTAC CTAACGCCGT TTACCTTTTG CCGTTGGTCC TCATTTCTCA AACCAACCAA 2820
ACCTCTCCCT CTTATAAAAT CCTCTCTCCC TTCTTTATTT CTTCCTCAGC AGCTTCTTCT 2880
GCTTTCAATT ACTCTCGCCG ACGATTTTCT CACCGGAAAA AAACAATATC ATTGCGGATA 2940
CACAAACTAT AATGGACCTC CTCTTGCTGG AGAAGTCTCT AATCGCCGTC TTCGTGGCGG 3000

TGATTCTCGC CACGGTGATT TCAAAGCTCC GCGGCAAGAA ATTGAAGCTA CCTCCAGGTC 3060

CTATACCAAT TCCGATCTTC GGAAACTGGC TTCAAGTAGG AGATGATCTC AACCACCGTA 3120

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ACCTAGTCGT CGTCTCTTCA CCGGATCTAA CCAAGGAAGT GCTCCACACA CAAGGCGTTG 3240

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TTTGCTTTTG GGAAATTGAC ATTGATGATT CATTCTTACA GGCAAATTGC GAGTTCTAAG 3840

CCTACAGGTA GTGAAGGATT GAAATGTGCC ATTGATCACA TCCTTGAAGC TGAGCAGAAG 3900

GGAGAAATCA ACGAGGACAA TGTTCTTTAC ATCGTCGAGA ACATCAATGT CGCCGGTAAC 3960

TTCTATTTCT TACTTGTAGG ATACGTAATC AATCCTCTAG ACGTCTCTGC TTGCATAAGG 4020

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TTGTGAAGCT GATATCTTGA TTTCTGCTGA AACAGCGATT GAGACAACAT TGTGGTCTAT 4200

CGAGTGGGGA ATTGCAGAGC TAGTGAACCA TCCTGAAATC CAGAGTAAGC TAAGGAACGA 4260

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ATACCTTCAA GCTGTGGTTA AGGAGACTCT TCGTCTGAGA ATGGCGATTC CTCTCCTCGT 4380

GCCTCACATG AACCTCCATG ATGCGAAGCT CGCTGGCTAC GATATCCCAG CAGAAAGCAA 4440

AATCCTTGTT AATGCTTGGT GGCTAGCAAA CAACCCCAAC AGCTGGAAGA AGCCTGAAGA 4500

GTTTAGACCA GAGAGGTTCT TTGAAGAAGA ATCGCACGTG GAAGCTAACG GAAATGACTT 4560

CAGGTATGTG CCGTTTGGTG TTGGACGTAG AAGCTGTCCC GGGATTATAT TGGCATTACC 4620

TATTTTGGGG ATCACCATTG GTAGGATGGT CCAGAACTTC GAGCTTCTTC CTCCTCCAGG 4680

ACAGTCTAAA GTGGATACTA GTGAGAAAGG TGGACAATTC AGCTTGCACA TCCTTAACCA 4740

CTCCATAATC GTTATGAAAC CAAGGAACTG TTAAACTTTC TGCACAAAAA AAAGGATGAA 4800

GATGACTTTA TAAATGTTTG TGAAATCTGT TGAAATATTC CCTTGTTTTG CTTTTGTGAG 4860

ATGTTTTTGT GTAAAATGTC TTTAAATGGT TCGTTCTACG ATTGCAATAA TAATTAGTGG 4920

TGCTCATTCT TTTGGATGGA TCGATGTTAT ACTTATATCA TTTGAAAATC TCATGATTGT 4980

TGGACTTGGA CCATAGTTGT TAATTTGAAG GTTTCTAGGT TCTAACGTTA ATAATCTTGT 5040

TCACACCAAA TAAATCTCAT TACACAATTT GGGGAGGTAT TAAAAGATTA CCAAAATAGG 5100

TTAATTACAA ATTCGACTAT TTCCAGTAAT ATGGGCTAAT ATAGGCTCCA ATTTAGATAC 5160

TAATAATGGG CTTTATAAAG CCCATTTGTT TTTCTCCTTA ATATCATCAC TCGCAGAGAT 5220

TACGCAGCGG GAATATAAAA ACACCAAATG CTTACAAGAA ATTTTCGAAA TTTGAAAGAC 5280

CGTTCGTTTC GTTGTCTTTG ATTTCCCCTG CTGCAAATTT GATCAAAGAT CATCGGATTC 5340

ATCATTCGGT AGCAGCAATT ATCATGTTCT CGTAATCGTT TCTATGCTCC GAGCTCCGTT 5400

TTGGGGACGC GATTCAGATA CTGTCGAAGC TT 5432

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1838 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AAAAAAAACA CTCAATATGG AGTCTTCTAT ATCACAAACA CTAAGCAAAC TATCAGATCC 60

CACGACGTCT CTTGTCATCG TTGTCTCTCT TTTCATCTTC ATCAGCTTCA TCACACGGCG 120

GCGAAGGCCT CCATATCCTC CCGGTCCACG AGGTTGGCCC ATCATAGGCA ACATGTTAAT 180

GATGGACCAA CTCACCCACC GTGGTTTAGC CAATTTAGCT AAAAAGTATG GCGGATTGTG 240

CCATCTCCGC ATGGGATTCC TCCATATGTA CGCTGTCTCA TCACCCGAGG TGGCTCGACA 300

AGTCCTTCAA GTCCAAGACA GCGTCTTCTC GAACCGGCCT GCAACTATAG CTATAAGCTA 360

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TCTGACTTAC GACCGAGCGG ACATGGCTTT CGCTCACTAC GGACCGTTTT GGAGACAGAT     420
GAGAAAAGTG TGTGTCATGA AGGTGTTTAG CCGTAAAAGA GCTGAGTCAT GGGCTTCAGT     480
TCGTGATGAA GTGGACAAAA TGGTCCGGTC GGTCTCTTGT AACGTTGGTA AGCCTATAAA     540
CGTCGGGGAG CAAATTTTTG CACTGACCCG CAACATAACT TACCGGGCAG CGTTTGGGTC     600
AGCCTGCGAG AAGGGACAAG ACGAGTTCAT AAGAATCTTA CAAGAGTTCT CTAAGCTTTT     660
TGGAGCCTTC AACGTAGCGG ATTTCATACC ATATTTCGGG TGGATCGATC CGCAAGGGAT     720
AAACAAGCGG CTCGTGAAGG CCCGTAATGA TCTAGACGGA TTTATTGACG ATATTATCGA     780
TGAACATATG AAGAAGAAGG AGAATCAAAA CGCTGTGGAT GATGGGGATG TTGTCGATAC     840
CGATATGGTT GATGATCTTC TTGCTTTTTA CAGTGAAGAG GCCAAATTAG TCAGTGAGAC     900
AGCGGATCTT CAAAATTCCA TCAAACTTAC CCGTGACAAT ATCAAAGCAA TCATCATGGA     960
CGTTATGTTT GGAGGAACGG AAACGGTAGC GTCGGCGATA GAGTGGGCCT TAACGGAGTT    1020
ATTACGGAGC CCCGAGGATC TAAAACGGGT CCAACAAGAA CTCGCCGAAG TCGTTGGACT    1080
TGACAGACGA GTTGAAGAAT CCGACATCGA GAAGTTGACT TATCTCAAAT GCACACTCAA    1140
AGAAACCCTA AGGATGCACC CACCGATCCC TCTCCTCCTC CACGAAACCG CGGAGGACAC    1200
TAGTATCGAC GGTTTCTTCA TTCCCAAGAA ATCTCGTGTG ATGATCAACG CGTTTGCCAT    1260
AGGACGCGAC CCAACCTCTT GGACTGACCC GGACACGTTT AGACCATCGA GGTTTTTGGA    1320
ACCGGGCGTA CCGGATTTCA AAGGGAGCAA TTTCGAGTTT ATACCGTTCG GGTCGGGTCG    1380
TAGATCGTGC CCGGGTATGC AACTAGGGTT ATACGCGCTT GACTTAGCCG TGGCTCATAT    1440
ATTACATTGC TTCACGTGGA AATTACCTGA TGGGATGAAA CCAAGTGAGC TCGACATGAA    1500
TGATGTGTTT GGTCTCACGG CTCCTAAAGC CACGCGGCTT TTCGCCGTGC CAACCACGCG    1560
CCTCATCTGT GCTCTTTAAG TTTATGGTTC GAGTCACGTG GCAGGGGGTT TGGTATGGTG    1620
AAAACTGAAA AGTTTGAAGT TGCCCTCATC GAGGATTTGT GGATGTCATA TGTATGTATG    1680
TGTATACACG TGTGTTCTGA TGAAAACAGA TTTGGCTCTT TGTTTGCCCT TTTTTTTTTT    1740
TTCTTTAATG GGGATTTTCC TTGAATGAAA TGTAACAGTA AAAATAAGAT TTTTTTCAAT    1800
AAGTAATTTA GCATGTTGCA AAAAAAAAAA AAAAAAAA                            1838
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5156 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AGTAACAAAT AGTGGTTTTA TTTTTTTAAA TATACAAAAA CTGTTTAACC ATTTTATTTC 120

TTGGTTAGCA AAATTTTGAT ATATTCTTAA GAAACTAATA TTTTAGGTTG ATATATTGCA 180

GTCACTAAAT AGTTTTAAAA GACACGAAGT TGGTAAGAAC AGGCATATAT TATTCGATTT 240

AATTAGGAAT GCTTATGTTA ATCTGATTCG ACTAATTAGA AACGACGATA CTATGAGCTC 300

ATAGATGGTC CCACGACCCA CTCTCCCATT TGATCAATAT TCAACTGAGC AATGAAACTA 360

ATTAAAAACG TGGTTAGATT AAAAAAATAA ATTGTGCAGG TAGCGGATAT ATAATACTAG 420

TAGGGGTTAA AAATAAAATA AAACACCACA GTATTAAATT TTTGTTTCAA AAGTATTATC 480

AATAGTTTTT TTGCTTCAAA AATATCACAA ATTTTTGTAT GAAATATTTC TTTAACGAAA 540

ATAAATTAAA TAAAATTTAA AATTTATATT TGGAGTTCTA TTTTTAATTT AGAGTTTTTA 600

TTGTTACCAC ATTTTTTGAA TTATTCTAAT ATTAATTTGT GATATTATTA CAAAAAGTAA 660

AAATATGATA TTTTAGAATA CTATTATCGA TATTTGATAT TATTGACCTT AGCTTTGTTT 720

GGGTGGAGAC ATGTGATTAT CTTATTACCT TTTTATTCCA TGAAACTACA GAGTTCGCCA 780

GGTACCATAC ATGCACACAC CCTCGTGAAG CCGTGACTTA ATATGATCTA GAACTTAAAT 840

AGTACTACTA ATTGTGTCAT TTGAACTTTC TCCTATGTCG GTTTCACTTC ATGTATCGCA 900

GAACAGGTGG AATACAGTGT CCTTGAGTTT CACCCAAATC GGTCCAATTT TGTGATATAT 960

ATTGCGATAC AGACATACAG CCTACAGAGT TTTGTCTTAG CCCACTGGTT GGCAAACGAA 1020

ATTGTCTTTA TTTTTTTATG TTTTGTTGTC AATGTGTCTT TGTTTTTAAC TAGATTGAGG 1080

TTTAATTTTA ATACATTTGT TAGTTTACAG ATTATGCAGT GTAATCTGAT AATGTAAGTT 1140

GAACTGCGTT GGTCAAAGTC TTGTGTAACG CACTGTATCT AAATTGTGAG TAACGACAAA 1200

ATAATTAAAA TTAAAGGACC TTCAAGTATT ATTAGTATCT CTGTCTAAGA TGCACAGGTA 1260

TTCAGTAATA GTAATAAATA ATTACTTGTA TAATTAATAT CTAATTAGTA AACCTTGTGT 1320
CTAAACCTAA ATGAGCATAA ATCCAAAAGC AAAAATCTAA ACCTAACTGA AAAAGTCATT 1380
ACGAAAAAAA GAAAAAAAAA AGAGAAAAAA CTACCTGAAA AGTCATGCAC AACGTTCATC 1440
TTGGCTAAAT TTATTTAGTT TATTAAATAC AAAAATGGCG AGTTTCTGGA GTTTGTTGAA 1500
AATATATTTG TTTAGCCACT TTAGAATTTC TTGTTTTAAT TTGTTATTAA GATATATCGA 1560
GATAATGCGT TTATATCACC AATATTTTTG CCAAACTAGT CCTATACAGT CATTTTTCAA 1620
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GCTCGAATTC AGTAAAATCC GTTTGGTATA CTATTTATTT CGTATAAGTA TGTAATTCCA 1740
CTAGATTTCC TTAAACTAAA TTATATATTT ACATAATTGT TTTCTTTAAA AGTCTACAAC 1800
AGTTATTAAG TTATAGGAAA TTATTTCTTT TATTTTTTTT TTTTTTTAGG AAATTATTTC 1860
TTTTGCAACA CATTTGTCGT TTGCAAACTT TTAAAAGAAA ATAAATGATT GTTATAATTG 1920
ATTACATTTC AGTTTATGAC AGATTTTTTT TATCTAACCT TTAATGTTTG TTTCCCTGTT 1980
TTTAGGAAAA TCATACCAAA ATATATTTGT GATCACAGTA AATCACGGAA TAGTTATGAC 2040
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ATCAAGATTT TAGGAAACGA TTTGAGCAAG GATTTAGAAG ATTTGAATCT TTAATTAAAT 2160
ATTTTCATTC CTAAATAATT AATGCTAGTG GCATAATATT GTAAATAAGT TCAAGTACAT 2220
GATTAATTTG TTAAAATGGT TGAAAAATAT ATATATGTAG ATTTTTTCAA AAGGTATACT 2280
AATTATTTTC ATATTTTCAA GAAAATATAA GAAATGGTGT GTACATATAT GGATGAAGAA 2340
ATTTAAGTAG ATAATACAAA AATGTCAAAA AAAGGGACCA CACAATTTGA TTATAAAACC 2400
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TGGGGATGTT GTCGATACCG ATATGGTTGA TGATCTTCTT GCTTTTTACA GTGAAGAGGC 3480
CAAATTAGTC AGTGAGACAG CGGATCTTCA AAATTCCATC AAACTTACCC GTGACAATAT 3540
CAAAGCAATC ATCATGGTAA TTATATTTCA AAAAGCACTA GTCATAGTCA TGTTTCTTAA 3600
TGCGTTACGT AATAATACTT ATCCATTGAC CAGTTATTTT CTCCTAAGTT TTTTTGTTTG 3660
AATTAGGAAG GTAATTTTCT ATTTTACTAG AGAAAGCAAC AGATTTTAGC ATGATCTTTT 3720
TTTAATATAT ATAGAAGCAT TGAATATTCA GATCTACAAT AATTATGAAA CTAATGAAGA 3780
GACAAAAAAT GGAGAGAGAA AAAAGAAAGA GTGGACTAGT GTGGATATAT TTAATTCTAA 3840
TTTGATTTTA TTAGGACGTT ATATTTAATT CTAATTTGAT TTTTTTATTT GATTTTATTA 3900
GGACGTTATG TTTGGAGGAA CGGAAACGGT AGCGTCGGCG ATAGAGTGGG CCTTAACGGA 3960
GTTATTACGG AGCCCCGAGG ATCTAAAACG GGTCCAACAA GAACTCGCCG AAGTCGTTGG 4020
ACTTGACAGA CGAGTTGAAG AATCCGACAT CGAGAAGTTG ACTTATCTCA AATGCACACT 4080
CAAAGAAACC CTAAGGATGC ACCCACCGAT CCCTCTCCTC CTCCACGAAA CCGCGGAGGA 4140
CACTAGTATC GACGGTTTCT TCATTCCCAA GAAATCTCGT GTGATGATCA ACGCGTTTGC 4200
CATAGGACGC GACCCAACCT CTTGGACTGA CCCGGACACG TTTAGACCAT CGAGGTTTTT 4260
GGAACCGGGC GTACCGGATT TCAAAGGGAG CAATTTCGAG TTTATACCGT TCGGGTCGGG 4320
TCGTAGATCG TGCCCGGGTA TGCAACTAGG GTTATACGCG CTTGACTTAG CCGTGGCTCA 4380
TATATTACAT TGCTTCACGT GGAAATTACC TGATGGGATG AAACCAAGTG AGCTCGACAT 4440
GAATGATGTG TTTGGTCTCA CGGCTCCTAA AGCCACGCGG CTTTTCGCCG TGCCAACCAC 4500

GCGCCTCATC TGTGCTCTTT AAGTTTATGG TTCGAGTCAC GTGGCAGGGG GTTTGGTATG 4560

GTGAAAACTG AAAAGTTTGA AGTTGCCCTC ATCGAGGATT TGTGGATGTC ATATGTATGT 4620

ATGTGTATAC ACGTGTGTTC TGATGAAAAC AGATTTGGCT CTTTGTTTGC CCTTTTTTTT 4680

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AATAAGTAAT TTAGCATGTT GCAAAGATCG ATCTTGGATG AGAACTTCTA CTTAAAAAAA 4800

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CTAAGGTTTT TATTAGTTTT ATTTTCAGTT TACTGAGTAC TATTTACTTT TTTATTTTTT 5040

GCAAATAAAT GTATTTTATC ATATTTATGT TTTTTGTTAT AAACTCCAAA CATACAGGTT 5100

TCATTACCTA AAAAAAGACA GAGTGGTTTC GTTAATTTTG TTTCATTAAT CTCGAG 5156

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 520 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu Ser Ser Ile Ser Gln Thr Leu Ser Lys Leu Ser Asp Pro Thr
1 5 10 15

Thr Ser Leu Val Ile Val Val Ser Leu Phe Ile Phe Ile Ser Phe Ile
20 25 30

Thr Arg Arg Arg Arg Pro Pro Tyr Pro Pro Gly Pro Arg Gly Trp Pro
35 40 45

Ile Ile Gly Asn Met Leu Met Met Asp Gln Leu Thr His Arg Gly Leu
50 55 60

Ala Asn Leu Ala Lys Lys Tyr Gly Gly Leu Cys His Leu Arg Met Gly
65 70 75 80

Phe Leu His Met Tyr Ala Val Ser Ser Pro Glu Val Ala Arg Gln Val
85 90 95

Leu Gln Val Gln Asp Ser Val Phe Ser Asn Arg Pro Ala Thr Ile Ala

100 105 110

Ile Ser Tyr Leu Thr Tyr Asp Arg Ala Asp Met Ala Phe Ala His Tyr
115 120 125

Gly Pro Phe Trp Arg Gln Met Arg Lys Val Cys Val Met Lys Val Phe
130 135 140

Ser Arg Lys Arg Ala Glu Ser Trp Ala Ser Val Arg Asp Glu Val Asp
145 150 155 160

Lys Met Val Arg Ser Val Ser Cys Asn Val Gly Lys Pro Ile Asn Val
165 170 175

Gly Glu Gln Ile Phe Ala Leu Thr Arg Asn Ile Thr Tyr Arg Ala Ala
180 185 190

Phe Gly Ser Ala Cys Glu Lys Gly Gln Asp Glu Phe Ile Arg Ile Leu
195 200 205

Gln Glu Phe Ser Lys Leu Phe Gly Ala Phe Asn Val Ala Asp Phe Ile
210 215 220

Pro Tyr Phe Gly Trp Ile Asp Pro Gln Gly Ile Asn Lys Arg Leu Val
225 230 235 240

Lys Ala Arg Asn Asp Leu Asp Gly Phe Ile Asp Asp Ile Ile Asp Glu
245 250 255

His Met Lys Lys Lys Glu Asn Gln Asn Ala Val Asp Asp Gly Asp Val
260 265 270

Val Asp Thr Asp Met Val Asp Asp Leu Leu Ala Phe Tyr Ser Glu Glu
275 280 285

Ala Lys Leu Val Ser Glu Thr Ala Asp Leu Gln Asn Ser Ile Lys Leu
290 295 300

Thr Arg Asp Asn Ile Lys Ala Ile Ile Met Asp Val Met Phe Gly Gly
305 310 315 320

Thr Glu Thr Val Ala Ser Ala Ile Glu Trp Ala Leu Thr Glu Leu Leu
325 330 335

Arg Ser Pro Glu Asp Leu Lys Arg Val Gln Gln Glu Leu Ala Glu Val
340 345 350

Val Gly Leu Asp Arg Arg Val Glu Glu Ser Asp Ile Glu Lys Leu Thr
355 360 365

Tyr Leu Lys Cys Thr Leu Lys Glu Thr Leu Arg Met His Pro Pro Ile
370 375 380

Pro Leu Leu Leu His Glu Thr Ala Glu Asp Thr Ser Ile Asp Gly Phe
385 390 395 400

```
Phe Ile Pro Lys Lys Ser Arg Val Met Ile Asn Ala Phe Ala Ile Gly
                405                 410                 415

Arg Asp Pro Thr Ser Trp Thr Asp Pro Asp Thr Phe Arg Pro Ser Arg
            420                 425                 430

Phe Leu Glu Pro Gly Val Pro Asp Phe Lys Gly Ser Asn Phe Glu Phe
        435                 440                 445

Ile Pro Phe Gly Ser Gly Arg Arg Ser Cys Pro Gly Met Gln Leu Gly
    450                 455                 460

Leu Tyr Ala Leu Asp Leu Ala Val Ala His Ile Leu His Cys Phe Thr
465                 470                 475                 480

Trp Lys Leu Pro Asp Gly Met Lys Pro Ser Glu Leu Asp Met Asn Asp
                485                 490                 495

Val Phe Gly Leu Thr Ala Pro Lys Ala Thr Arg Leu Phe Ala Val Pro
            500                 505                 510

Thr Thr Arg Leu Ile Cys Ala Leu
        515                 520
```

What is claimed is:

1. An isolated DNA construct comprising a tissue-specific regulatory plant promoter operably linked to a nucleotide sequence encoding an enzyme of the phenylpropanoid pathway;

wherein the promoter regulates expression of the nucleotide sequence in a host plant cell; and

wherein the host plant cell expresses the nucleotide sequence.

2. The DNA construct according to claim 1, wherein the enzyme is selected from the group consisting of phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), caffeic acid/ 5-hydroxyferulic acid O-methyltransferase (OMT), ferulate-5-hydroxylase (F5H), (hydroxy)cinnamoyl-CoA ligase (4CL), (hydroxy)cinnamoyl-CoA reductase (CCR), (hydroxy)cinnamoyl alcohol dehydrogenase (CAD), laccase, and enzymes having substantial identity thereto.

3. The DNA construct according to claim 1, wherein the enzyme is a ferulate-5-hydroxylase (F5H) enzyme.

4. The DNA construct according to claim 1, wherein the promoter is selected from the group consisting of phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), caffeic acid/ 5-hydroxyferulic acid O-methyltransferase (OMT), (hydroxy)cinnamoyl-CoA ligase (4CL), (hydroxy)cinnamoyl-CoA reductase (CCR), (hydroxy)cinnamoyl alcohol dehydrogenase (CAD), and laccase.

5. The DNA construct according to claim 1, wherein the promoter is a cinnamate-4-hydroxylase (C4H) promoter.

6. A vector useful for transforming a cell, said vector comprising a tissue-specific

regulatory plant promoter operably linked to a nucleotide sequence encoding a ferulate-5-hydroxylase (F5H) enzyme;

wherein the promoter regulates expression of the nucleotide sequence in a host plant cell.

7. A plant transformed with the vector of claim 6, or progeny thereof, the plant being capable of expressing the nucleotide sequence.

8. The plant according to claim 7, the plant being selected from the group consisting of alfalfa *(Medicago* sp.), rice *(Oryza* sp.), maize *(Zea mays)*, oil seed rape *(Brassica* sp.), forage grasses, and also tree crops such as eucalyptus *(Eucalyptus* sp.), pine *(Pinus* sp.), spruce *(Picea* sp.) and poplar *(Populus* sp.), as well as *Arabidopsis* sp. and tobacco *(Nicotiana* sp.).

9. The plant according to claim 7, wherein the plant produces lignin having a syringyl monomer content greater than the syringyl content of lignin produced by a non-transformed plant of the same species.

10. A method for increasing the syringyl content of lignin in a target plant, comprising: providing a vector comprising a tissue-specific regulatory plant promoter operably linked to a nucleotide sequence encoding a ferulate-5-hydroxylase (F5H) enzyme; wherein the promoter regulates expression of the nucleotide sequence in a host plant cell; and

transforming the target plant with the vector to provide a transformed plant, the transformed plant being capable of expressing the nucleotide sequence.

11. The method according to claim 10, wherein the enzyme comprises an amino acid sequence having substantial identity to the sequence set forth in SEQ ID NO: 4.

12. The method according to claim 10, wherein the transformed plant produces lignin

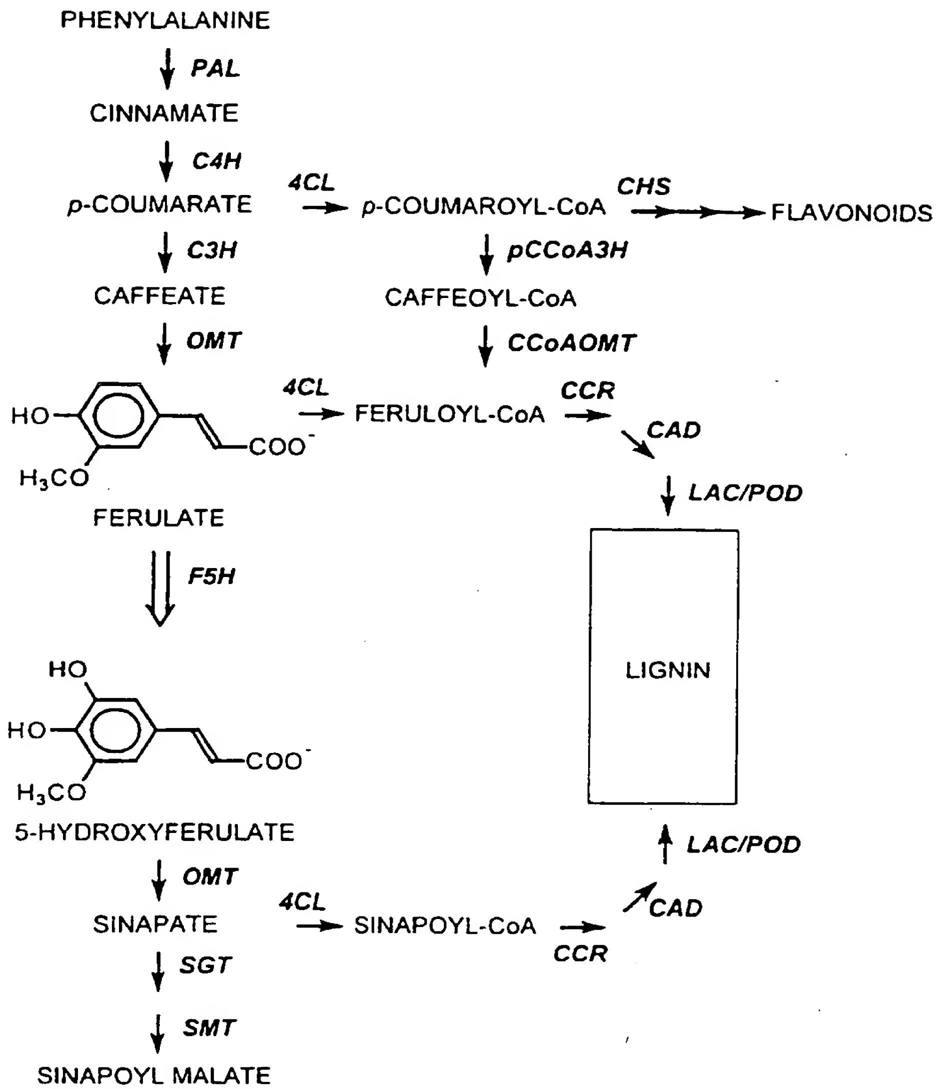
having a syringyl monomer content greater than the syringyl content of lignin produced by a non-transformed plant of the same species.

13. The method according to claim 10, wherein the target plant is selected from the group consisting of alfalfa *(Medicago* sp.), rice *(Oryza* sp.), maize *(Zea mays)*, oil seed rape *(Brassica* sp.), forage grasses, and also tree crops such as eucalyptus *(Eucalyptus* sp.), pine *(Pinus* sp.), spruce *(Picea* sp.) and poplar *(Populus* sp.), as well as *Arabidopsis* sp. and tobacco *(Nicotiana* sp.).

14. The method according to claim 10, wherein the nucleotide sequence has substantial identity to the nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:3.

15. A transgenic plant obtained according to the method of claim 10 or progeny thereof.

16. A method of producing a transformed plant, comprising incorporating into the nuclear genome of the plant an isolated nucleotide sequence which encodes an enzyme comprising an amino acid sequence having substantial identity to the sequence set forth in SEQ ID NO: 4, to provide a transformed plant capable of expressing the enzyme in an amount effective to increase the syringyl content of the plant's lignin composition.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PAL
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p-COUMARATE
4CL
p-COUMAROYL-CoA
CHS
FLAVONOIDS
C3H
pCCoA3H
CAFFEATE
CAFFEOYL-CoA
OMT
CCoAOMT
HO
H3CO
COO-
4CL
FERULOYL-CoA
CCR
CAD
FERULATE
LAC/POD
F5H
LIGNIN
HO
HO
H3CO
COO-
5-HYDROXYFERULATE
LAC/POD
OMT
CAD
4CL
SINAPATE
SINAPOYL-CoA
CCR
SGT
SMT
SINAPOYL MALATE

Figure 1

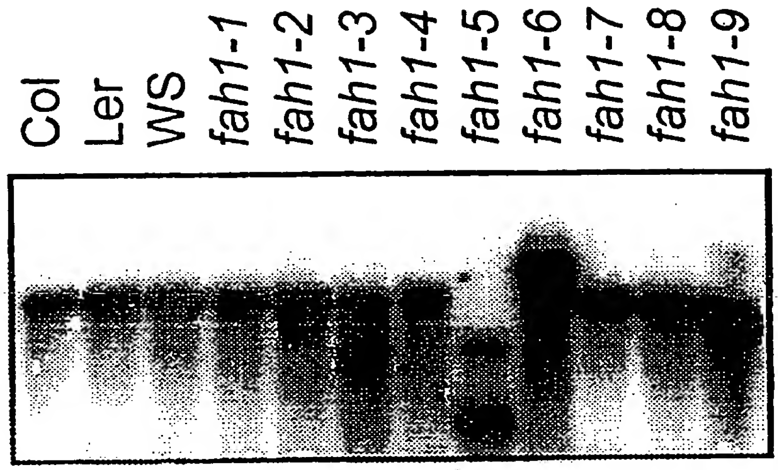
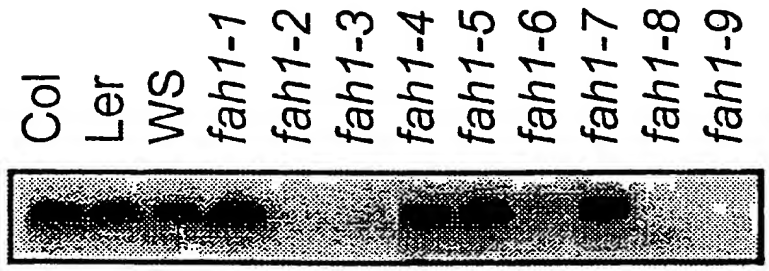

Col
Ler
WS
fah1-1
fah1-2
fah1-3
fah1-4
fah1-5
fah1-6
fah1-7
fah1-8
fah1-9

Figure 2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WS
fah1-1
fah1-2
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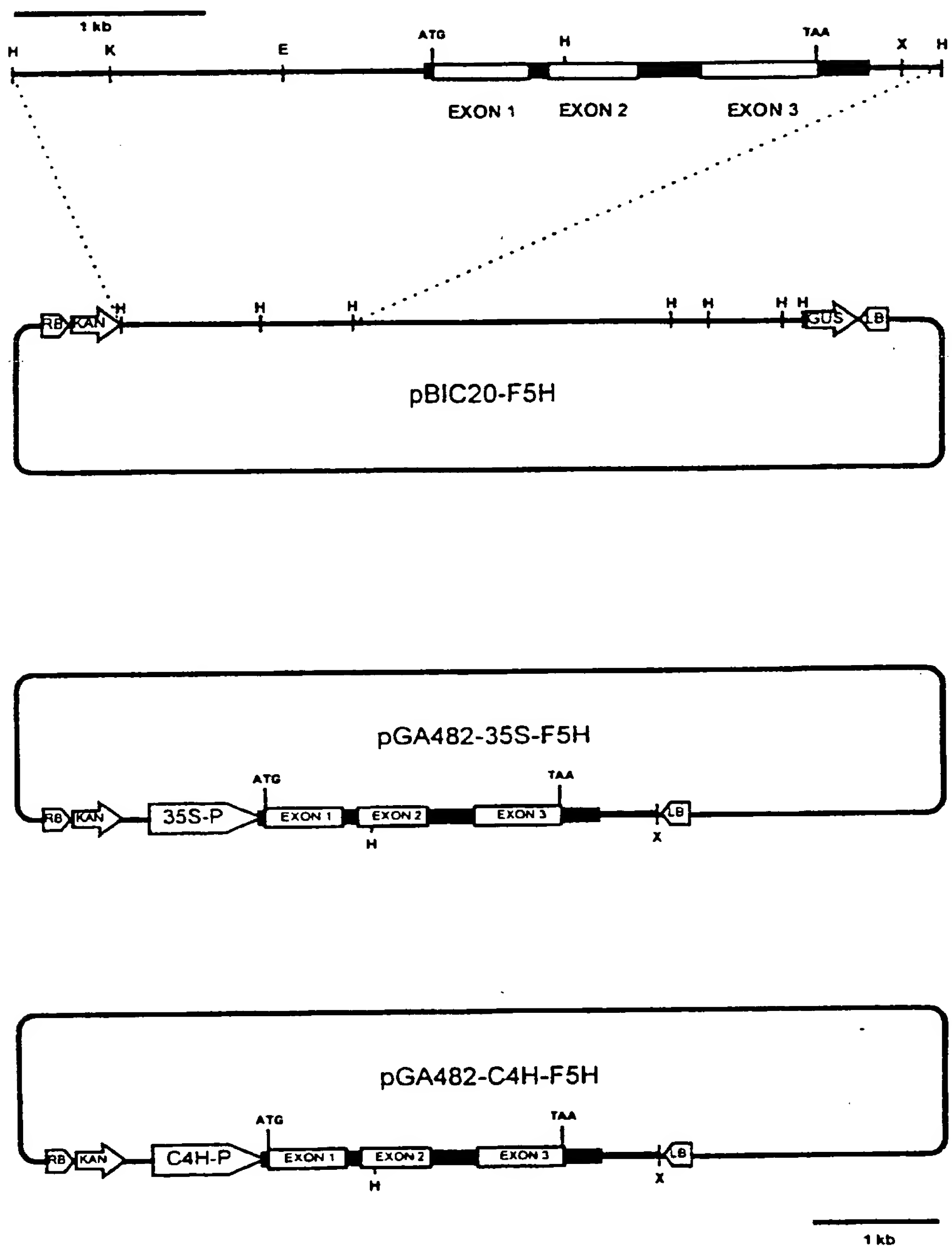
fah1-4
fah1-5
fah1-6
fah1-7
fah1-8
fah1-9

Figure 3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H
K
E
ATG
H
TAA
X
H
EXON 1
EXON 2
EXON 3
RB
KAN
GUS
LB
pBIC20-F5H
pGA482-35S-F5H
35S-P
pGA482-C4H-F5H
C4H-P

Figure 4

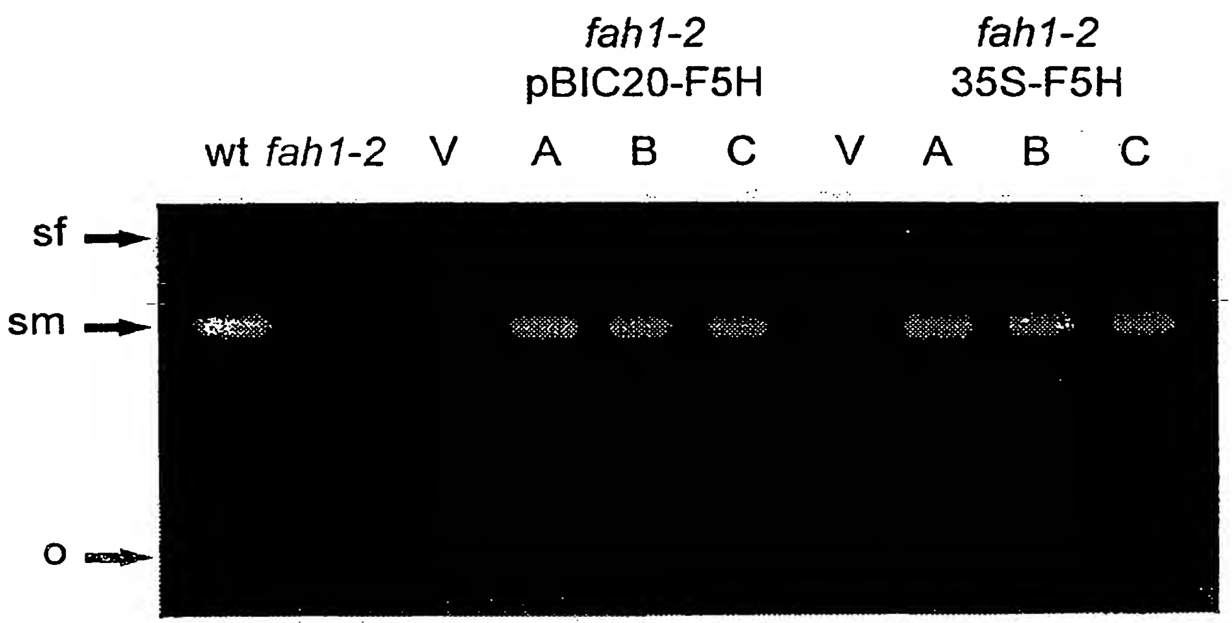


Figure 5

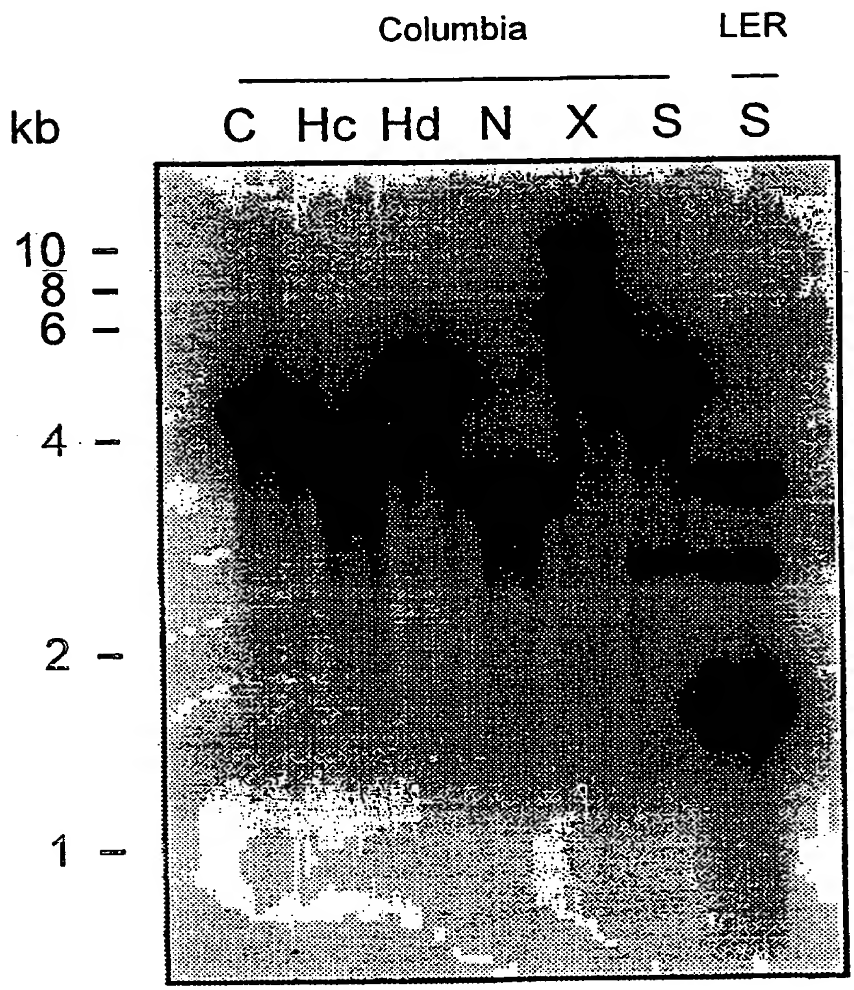


Figure 6

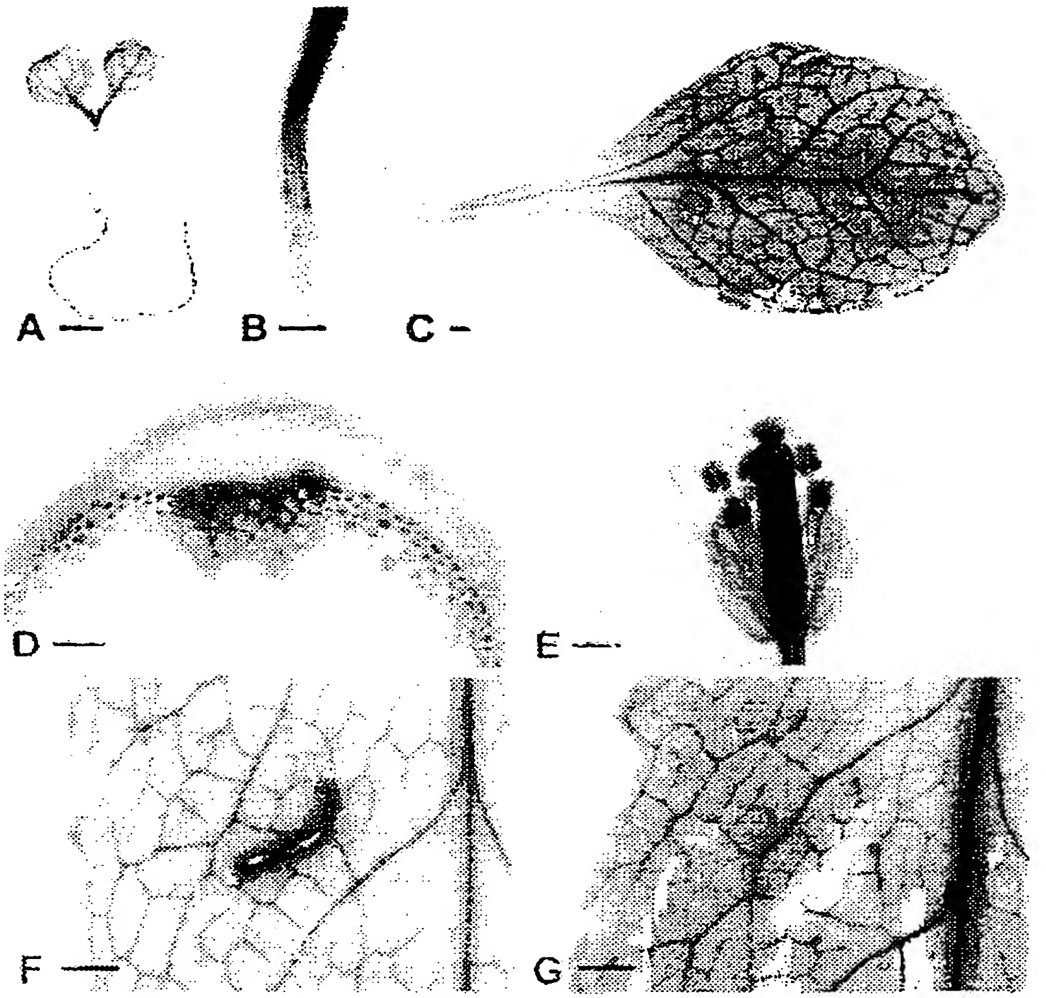
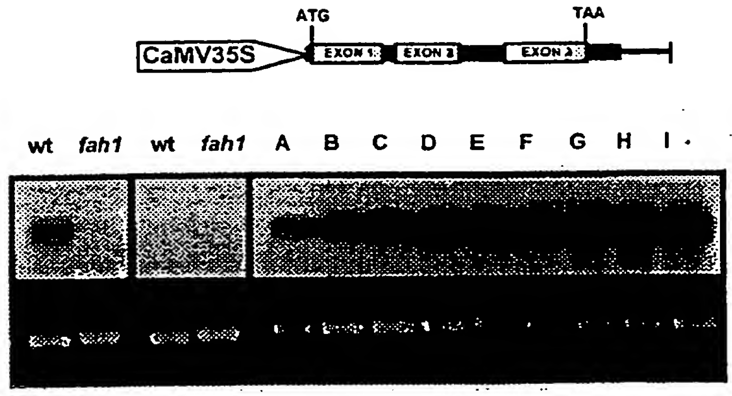


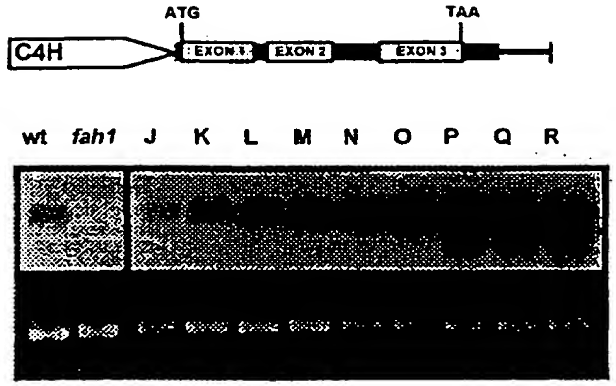
Figure 7



	wt	*fah1*	wt	*fah1*	A	B	C	D	E	F	G	H	I
mol% S			18	n.d.	5.1	13	19	20	23	25	26	29	28
std. dev.			0.9		0.2	0.6	0.6	0.9	0.8	1.2	0.8	0.9	1.8

Figure 8

Figure 9



	wt	fah1	J	K	L	M	N	O	P	Q	R
mol% S	20	n.d.	83	48	45	71	90	78	85	92	90
std. dev.	2.3		1.0	1.0	1.6	1.9	0.3	2.0	0.8	0.4	0.5

Figure 10

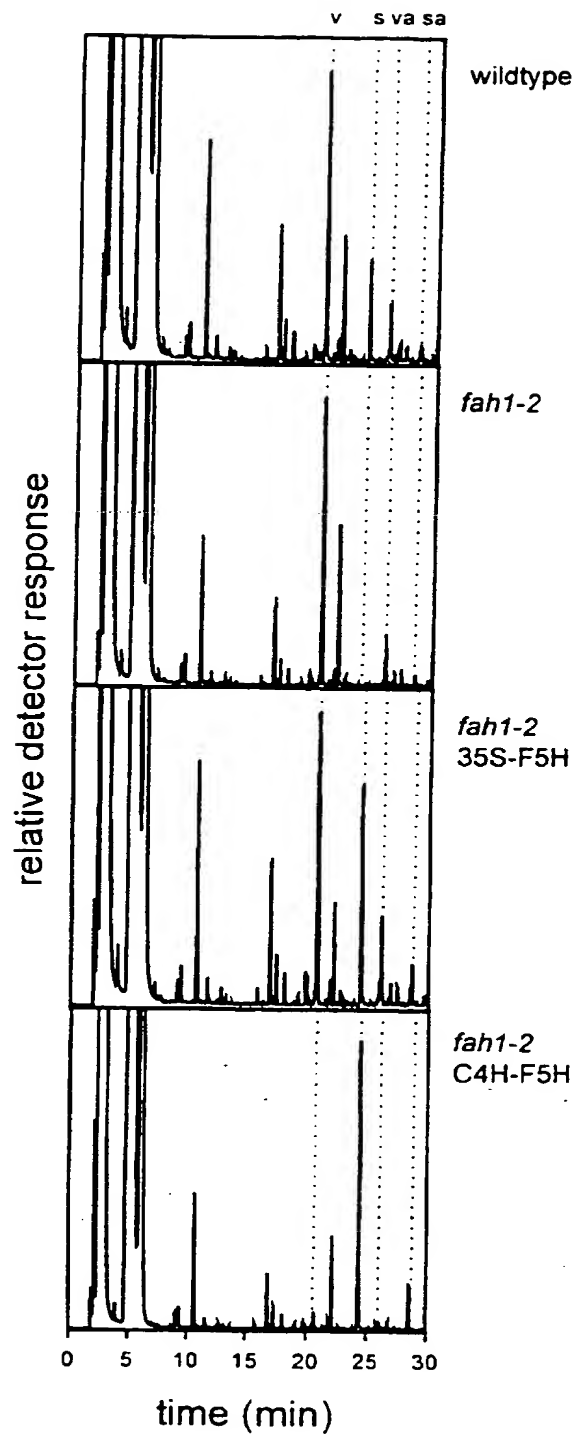

v
s va sa
wildtype
fah1-2
fah1-2
35S-F5H
fah1-2
C4H-F5H
relative detector response
0
5
10
15
20
25
30
time (min)

Figure 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12624

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12N 5/04, 5/10, 15/01, 15/10, 15/82, 15/83, 15/84
US CL :435/172.3, 320.1, 410, 419; 536/23.1, 23.2, 24.1; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1, 410, 419; 536/23.1, 23.2, 24.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Medline, Biosis, NTIS, biotech
Search terms: phenylpropanoid, ferulate-5-hydroxylase, promoter, syringyl, lignin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,451,514 A (BOUDET et al.) 19 September 1995, columns 1-2, claims 1-6.	1-16
A	DWIVEDI et al. Modification of Lignin Biosynthesis in Transgenic Nicotiana Through Expression of an Antisense o-Methyltransferase Gene from Populus. Plant Molecular Biology. 1994, Vol. 26, pages 61-71, especially page 69.	1-16
A	GOFFNER et al. Purification and Characterization of Isoforms of Cinnamyl Alcohol Dehydrogenase from Eucalyptus Xylem. Planta. 1992, Vol. 188, pages 48-53, especially pages 52-53.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 04 SEPTEMBER 1997	Date of mailing of the international search report 17 OCT 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer David Guzo JaB for Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GRAND, C. Ferulic Acid 5-Hydroxylase: A New Cytochrome P-450-Dependent Enzyme from Higher Plant Microsomes Involved in Lignin Synthesis. FEBS Letters. April 1994, Vol. 169, No. 1, pages 7-11, especially page 11.	1-16